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SHREYA SAHA

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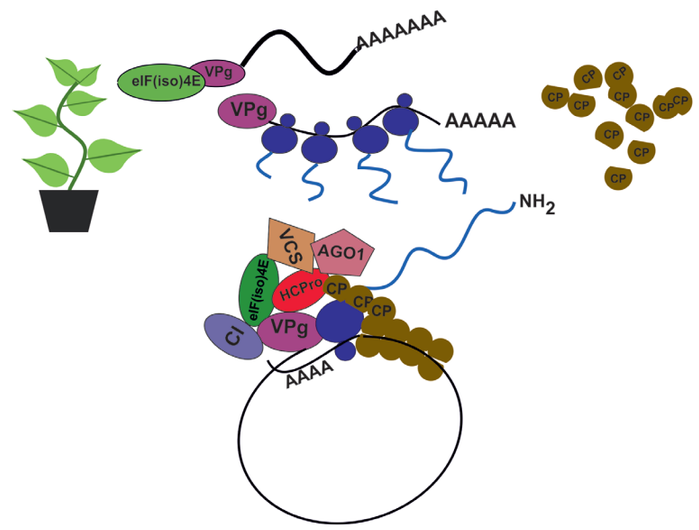

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VPg-eIF(iso)4E interaction, coat protein production and virion formation in potato virus A infection

Shreya Saha

ACADEMIC DISSERTATION
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Cover image- Host eIf(iso)4E and PVA VPg interaction, enhanced coat protein production via translation, coat protein binding to the PVA RNA, and accumulation of different host and viral proteins at the 5' end of PVA RNA to initiate stable virion formation. The image is prepared by Shreya Saha.
Cover layout- Anita Tienhaara

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Table of Contents

LIST OF ORIGINAL PUBLICATIONS

Saha, S., Hafren, A., and Mäkinen K. 2019. Dynamics of protein accumulation from the 3' end of viral RNA are different from those in the rest of the genome in potato virus A infection. *Journal of Virology,* 93:e00721-19. https://doi.org/10.1128/JVI.00721-19.

Saha, S., and Mäkinen K. 2020. Insights into the functions of eIF4E-binding motif of VPg in potato virus A infection" Viruses. 12, 197.

Andres Lõhmus*, Shreya Saha*, Pinky Dutta, Maija Pollari and Kristiina Mäkinen. Insights into the functions of HCPro and CP in RNA silencing suppression, viral protein accumulation and virion formation in potato virus A infection. *Manuscript.*

Author's contribution

Paper I: Shreya Saha participated in designing the experiments and executed major part of them (those presented in Figures 1-8). She was involved in interpretation of the data, prepared the figures for the paper and wrote the first draft of the MS.

Paper II: Shreya Saha participated in designing the experiments and carried out them. She was involved in interpretation of the data, prepared the figures for the paper and wrote the first draft of the MS.

Manuscript III: Shreya Saha participated in designing the experiments. She executed the experiments presented in Figures 3 and 5. She prepared those figures and contributed to writing of the manuscript. Shreya Saha and Andres Lõhmus had an equal contribution to this study.

ABBREVIATIONS

(-) -strand –minus-strand
(+) -strand – plus-strand
AGO1– argonaute 1
Avr– avirulence factor
BMV– brome mosaic virus
CHIP – C-terminus of Hsc70 interacting protein
CI –cylindrical inclusion protein
CP – coat protein
CPIP – coat protein-binding protein
dpi – days post infiltration
dsRNA – double-stranded RNA
eEF1A – eukaryotic translation elongation factor 1A
eIF4A – eukaryotic translation initiation factor 4A
eIF4E/eIF(iso)4E–eukaryotic translation initiation factor 4E/eukaryotic translation initiation factor (iso)4E
eIF2β, 3b– eukaryotic translation initiation factor 2β, 3b
ELISA– enzyme -linked immunosorbent assay
EM – electron microscopy
FLUC – firefly luciferase
ER – endoplasmic reticulum
GFP – green fluorescent protein
gRNA – genomic RNA
GUS– glucorunidase
HCPro – helper component-proteinase
HIV – human immunedeficiency virus
HSP70/HSP40 – heat shock protein 70/heat shock protein 40
icDNA – infectious complementary DNA
IC-RT-qPCR – immunocapture-RT-qPCR
IRES – internal ribosome entry site
kDa – kilodalton
LMV – lettuce mosaic virus
mRNA– messenger RNA
m7GpppG– methylguanosine-(5′) triphosphate (5′) guanosine
NIa – nuclear inclusion protein a
NIb – nuclear inclusion protein b
NMR– nuclear magnetic resonance

ORF – open reading frame
P0 – acidic ribosomal protein P0
P19 – tombusviral protein P19
P25 – potexviral protein P25
P2b - cucumoviral protein 2b
PABP – polyadenylate binding protein
PD – plasmodesmata
PG – PVA-induced granule
PIPO – pretty interesting potyvirus ORF
PPV – plum pox virus
PVA – potato virus A
PVX – potato virus X
PVY – potato virus Y
PVBV– pepper vein banding virus
RT-qPCR –quantitative real-time polymerase chain reaction
R-gene – resistance gene
RBP– RNA binding protein
RdRP – RNA-dependent RNA polymerase
RFP – red fluorescent protein
RLUC/*Rluc*– *Renilla* luciferase protein/gene
5'RLUC – *Renilla* luciferase protein produced from the 5'site of PVA RNA
3'RLUC- *Renilla* luciferase protein produced from the 3'site of PVA RNA
RNP – ribonucleoprotein
sgRNA – subgenomic RNA
ssRNA – single-stranded RNA
TEV – tobacco etch virus
TuMV – turnip mosaic virus
UBP1 – oligouridylate binding protein 1
UTR – untranslated region
VCS – varicose
VPg – viral protein genome-linked
VRC – viral replication complex
vRNA – viral RNA
VSR – viral suppressor of RNA silencing
WT – wild type
YFP – yellow fluorescent protein

The standard abbreviations for nucleotides and amino acids are used.

ABSTRACT

Potato Virus A (PVA), which belongs to the family *Potyviridae*, is a significant agricultural plant virus that causes crop loss worldwide. Most potyvirus resistance is recessive and occurs due to the loss of interaction between the viral protein genome-linked (VPg) and the host eukaryotic initiation factor [eIF4E/(iso)4E]. This interaction has been demonstrated in many cultivated plants that are susceptible to potyviruses. Studies on potyvirus resistance have shown that minute changes in either eIF4E/(iso)4E or VPg can cause the interaction to fail, resulting in the development of viral resistance. However, the detailed mechanisms underlying the significant effects of this interaction during potyvirus infection remain unclear. The central domain of the PVA VPg contains an eIF(iso)4E-binding consensus motif, Tyr-X-X-X-X-Leu-phi (YXXXLΦ). The function of this motif during the VPg–eIF(iso)4E interaction, in the context of PVA infection, was investigated in the present study. The tyrosine and the leucine residues at the binding site were replaced with alanine residues in PVA infectious cDNA (icDNA, PVA^{VPgmut}) and in a VPg expression construct (VPg^{mut}). The results showed that PVA^{VPgmut} was capable of replicating inside the host cell, but overall gene expression remained low, similar to the levels observed for a replication-deficient virus. Systemic infection in PVA^{VPgmut}-infected plants only occurred upon reversion to the wild-type PVA, which occurred in 26% of PVA^{VPgmut}-infected plants by 15 days postinfection. Although VPg typically stabilises viral RNA (vRNA) and 3' renilla luciferase (RLUC) expression, as published previously, the VPg^{mut} failed to perform these functions. The helper component proteinase (HCPro) induces the generation of PVA-induced granules (PGs) during infection and the assembly of vRNA within these PGs to safeguard vRNA from being silenced. Plants infected with PVA^{VPgmut} showed an increased number of PG-like foci in infected cells as compared to the plants infected with PVA^{WT}. However, in compare to the PVA^{WT} the percentage of PVA RNA colocalising with PGs was significantly low in PVA^{VPgmut} infected leaf samples. The host eIF(iso)4E is thought to bind to the PVA VPg via the YXXXLΦ motif, and this interaction is considered to be essential for PVA RNA stabilisation, the transfer of RNA to the RNA silencing suppression pathway, and RNA translocation to polysomes for viral protein synthesis.

In the second study, an alternative mechanism was explored involving the production of large quantities of coat protein (CP), which is a multi-functional protein. Tight control over CP production is necessary, depending on the stage of

virus infection. Increased CP concentrations have been shown to represses potyviral gene expression. Therefore, CP concentrations are maintained at low levels during active gene expression in the early infection stage through a host-mediated degradation system. During later infection stages, CP is required at high quantities for the production of a large number of stable particles. The present study showed that ectopically expressed VPg enhances reporter expression more pronouncedly from the 3' side of the genome than from the 5’ side. A similar phenomenon was observed towards the later stages of the infection, in which the 3’ CP cistron and the 3’ reporter cistron were expressed more pronouncedly than the central cylindrical inclusion (CI) cistron and the 5’ reporter cistron. The 3’CP and 3’ reporter protein showed different production/accumulation dynamics than were observed for the rest of the genome. CP expression levels were observed to increase in the presence of overexpressed VPg, during both the early infection stage and towards the later infection stage. This process could represent the mechanism through which potyviruses increase CP production for sufficient virion formation.

In the third study of this thesis, whether the stabilisation of CP was necessary for successful virion formation was investigated. These results revealed the function of PVA HCPro during CP stabilisation and virion formation. HCPro was found to be unable to stabilise CP in a virus-free system. A number of additional host and viral factors are necessary to produce stable particles. CP stability by HCPro is unrelated to its capacity for silencing suppression and, therefore, cannot be complemented by other viral silencing suppressors. Together, the findings described in this dissertation revealed important mechanisms that underlie potyvirus recessive resistance and the factors that affect stable virion formation.

1.INTRODUCTION

Approximately 15% of global crop production is affected by plant diseases, one-third of which are caused by plant viruses (Boualem et al., 2016). Unlike many other pathogens, plant viruses are intracellular obligate parasites and cannot be controlled by chemicals or pesticides. The most useful preventative method for viral plant diseases is the use of virus-resistant cultivars, virus-free planting materials, and appropriate agricultural practices for virus control (Jones, 2006). Viruses present unique features that distinguish them from other pathosystems, including the minimal number of proteins produced by viruses, a predominant dependence on the host cellular machinery, and the use of RNA silencing mechanism as a primary defence. Most viral proteins are multifunctional, and their activities and production are tightly regulated. Plant virus produce a large number of stable, transmissible particles as a consequence of successful infection. A high viral titre is essential for cell-to-cell transmission (Gutiérrez et al., 2012) and for aphid-mediated interhost transmission (Kaur et al., 2016; Shi et al., 2016). This dissertation focuses on and explores three aspects of potyvirus biology. First, the molecular mechanisms that underly recessive eukaryotic initiation factor 4E (eIF4E)-based resistance against potyvirus infection were scrutinised. Second, the mechanisms that regulate coat protein (CP) production for virion formation were examined. Third, the factors involved in CP stability and particle formation were investigated.

1.1 Potyvirus

1.1.1 Positive-sense, single-stranded (+ss) RNA viruses belonging to the Picorna-like subgroup of RNA viruses

In 1971, David Baltimore classified viruses into seven groups based on their genome type and method of replication. Positive-sense, single-stranded (+ss) RNA viruses were categorised to group IV, defined as single-stranded RNA viruses whose mRNA is identical to its virion RNA. +RNA viruses can infect Bactria and Eukarya but not Archaea (Nasir et al., 2014). More than half of all plant viral pathogens are +RNA viruses (Nasir et al., 2014). Based on the amino acid sequence of RNA-dependent RNA polymerase (RdRP), +RNA viruses are

categorised into three groups; Picorna-like, Flavi-like, and Alpha-like viruses (Goldbach, 1987; Goldbach and Wellink, 1988; Koonin and Dolja, 1993). The genome size of Picorna-like viruses can vary between 2–32 kb. The Picorna-like superfamily is characterised by (i) a positive-sense RNA genome, (ii) RdRP expression, (iii) the chymotrypsin-like protease superfamily 3 helicase (S3H), (iv) a genome-linked protein, (Koonin et al., 2008), and (v) icosahedral virions, featuring capsid proteins with a characteristic jell-roll fold. Potyvirus belongs to the family *Potyviridae* in the Picorna-like superfamily. However, the *Potyviridae* family is exceptional among Picorna-virus family members because it encodes a different capsid protein, features filamentous virions, and expresses a superfamily 2 helicase instead of S3H. Therefore, although the family *Potyviridae* is categorised as a member of the Picorna-like superfamily, it is excluded from the order *Picornavirales* (Koonin et al., 2008; Olendraite et al., 2017).

Potyvirus is the largest genus of the *Potyviridae* family (Fauquet and Fargette, 2005) and infect a wide range of plant species, including a large number of crop plants. Potyviral members can be found worldwide, transmitted by aphids, in a nonpersistent manner, seeds, or infected living plant materials (Pirone and Blanc, 1996). Potyviruses have flexuous, filamentous particles that are 700–750 nm long, 11-15 nm wide, and composed of 2,000 units of CP monomer (Carrington and Dougherty, 1988). The potyviral genome contains a single, large open reading frame (ORF), which is translated into a single large polyprotein, and a smaller overlapping ORF, called pretty interesting potyvirus ORF (PIPO) (Chung et al., 2008), is embedded within the P3 cistron. The large polyprotein is cleaved into at least ten proteins by virus-encoded proteinases produced by the polyprotein (Cui and Wang, 2016; Revers and García, 2015; White, 2015). Interestingly, PIPO is produced from the viral genome by a frame-shift mechanism, which is mediated by polymerase slippage on a GA6 site (Olspert et al., 2015).

Table.1 Potyviral proteins and their major roles in infection

Protein	Brief description and functions	References
P1	a serine protease with self-cleavable properties at the C terminal side, separates itself from the rest of the polyprotein	(Verchot et al., 1991)
	genome amplification	(Verchot and Carrington, 1995)

	host specificity, enhance infectivity of plum pox virus (PPV)	(Pasin et al., 2014)
Helper component protease (HCPro)	cysteine protease with self-cleavable properties at the C- terminal side, separates HCPro from the rest of the polyprotein	(Carrington et al., 1989)
	essential factor for aphid-mediated plant-plant transmission	(Govier and Kassanis, 1974a, b; Govier et al., 1977)
	silencing suppression	(Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998)
	systemic movement	(Cronin et al., 1995; Kasschau et al., 1997)
	synergistic interaction with other viruses	(González-Jara et al., 2005; Shi et al., 1997)
	facilitates CP stabilization and particle encapsidation.	(Valli et al., 2014)
P3	involved in viral replication	(Klein et al., 1994)
	symptom and avirulence determinant	(Jenner et al., 2003)
	viral infectivity	(Luan et al., 2016)
P3N-PIPO	generated due to transcriptional slippage, embeded within P3 cistron.	(Chung et al., 2008; Olspert et al., 2015)
	is involved in virus movement, act as an anchor in plasmodesmata channel used for viral movement	(Vijayapalani et al., 2012)
Six kilo dalton protein 1(6K1)	one of the smallest potyviral proteins, involved in viral replication	(Cui and Wang, 2016)
	Undefined role in potyvirus multiplication	(Kekarainen et al., 2002; Merits et al., 2002)
Cylindrical inclusion protein (CI)	produces potyviral signature, pinwheel structures, into cytoplasm (cylindrical inclusion)	(Roberts et al., 1998)
	has ATPase and RNA helicase activities and is involved in viral replication	(Eagles et al., 1994; Fernández et al., 1997; Laín et al., 1991; Laín et al., 1990)
	is involved in viral movement along with P3N-PIPO	(Rodríguez-Cerezo et al., 1997; Wei et al., 2010b)

	interacts with several of host and viral factors and associates with 5' tip structure of potyviral virions.	(Bilgin et al., 2003; Bosque et al., 2014; Elena and Rodrigo, 2012; Gabrenaite-Verkhovskaya et al., 2008; Jiménez et al., 2006; Tavert-Roudet et al., 2012; Torrance et al., 2006)
	acts as virulence factor for different resistance genes	(Sorel et al., 2014)
Six kilodalton protein 2 (6K2)	small membrane-associated protein involved in cellular endomembrane re-modelling to initiate virus replication, vesicles production.	(Cotton et al., 2009; Schaad et al., 1997a)
	involved in long distance movement and symptom development	(Spetz and Valkonen, 2004)
Virus protein genome linked (VPg)	is disordered molten globule protein with many interacting host and viral proteins and acts as a major virulence determinant in potyvirus infection	(Jiang and Laliberté, 2011; Oruetxebarria et al., 2001; Rantalainen et al., 2011; Rantalainen et al., 2008; Wang and Krishnaswamy, 2012)
	remains covalently attached to the 5' end of potyviral genome and acts as a primer to initiate replication	(Anindya et al., 2005; Oruetxebarria et al., 2001; Puustinen and Mäkinen, 2004)
	is involved in initiation of translation. Enhance viral translation and prevent host cellular translation.	(Beauchemin et al., 2007; Eskelin et al., 2011; Hafrén et al., 2013; Hafrén et al., 2015; Léonard et al., 2000; Léonard et al., 2004; Wittmann et al., 1997)
	protect potyvirus granules from autophagy	(Hafrén et al., 2018)
	suppresses sense-mediated RNA silencing by interacting with SGS3	(Rajamäki et al., 2014)

Nuclear inclusion protein a (NIa-Pro)	a protease responsible for the cleavage of individual proteins from the central to the C-terminal region of potyviral polyprotein	(Adams et al., 2005)
	Possess DNase activity, speculated to degrade host DNA in the nucleus which could play some regulatory role in host gene expression involved in viral infection	(Anindya and Savithri, 2003)
	interacts with a wide range of Arabidopsis host proteins, regulates biotic and abiotic stress, photosynthesis, metabolism and ethylene-mediated defense response	(Martínez et al., 2016)
Nuclear inclusion protein b (NIb)	RNA-dependent RNA polymerase (RDRP) responsible for viral replication	(Hong and Hunt, 1996)
	Uridylylate VPg protein, essential for replication	(Anindya et al., 2005; Puustinen and Mäkinen, 2004)
	interacts with eEF1A, PABP, heat shock protein Hsc70-3 required to form viral replication complex	(Dufresne et al., 2008; Thivierge et al., 2008; Wang et al., 2000)
	Interacts with SUMO-conjugated enzyme SCE1 in both nucleus and cytoplasm, important for potyvirus infection	(Xiong and Wang, 2013)
Coat protein (CP)	encapsidates of potyviral RNA. Is known to form virus like particles even in absence of viral RNA (vRNA)	(Dolja et al., 1994; Jagadish et al., 1991; Varrelmann and Maiss, 2000; Voloudakis et al., 2004)
	is involved in viral gene expression; regulation of replication and translation	(Besong-Ndika et al., 2015; Hafrén et al., 2010; Ivanov et al., 2003; Ivanov et al., 2001; Lõhmus et al., 2017)
	Involved in viral cell-to-cell movement	(Anindya and Savithri, 2003; Dolja et al., 1994; Ivanov et al., 2003; Ivanov et al., 2001)

1.1.2 Potato Virus A (PVA)

Potato virus A (PVA) was used as a model potyvirus in this study. PVA particles are approximately 700 nm long and 15 nm wide (Fribourg and De Zoeten, 1970). Similar to other potyviruses, the PVA genome contains one large ORF (Puurand et al., 1994) and one small PIPO (Chung et al., 2008). The product of the large ORF is proteolytically processed by three virus-encoded proteinases to produce ten mature proteins (Merits et al., 2002), and the PIPO produces the protein P3N-PIPO (Chung et al., 2008). A schematic showing the genome organisation of PVA and an electron microscopy image of PVA particles are presented in Figure 1.

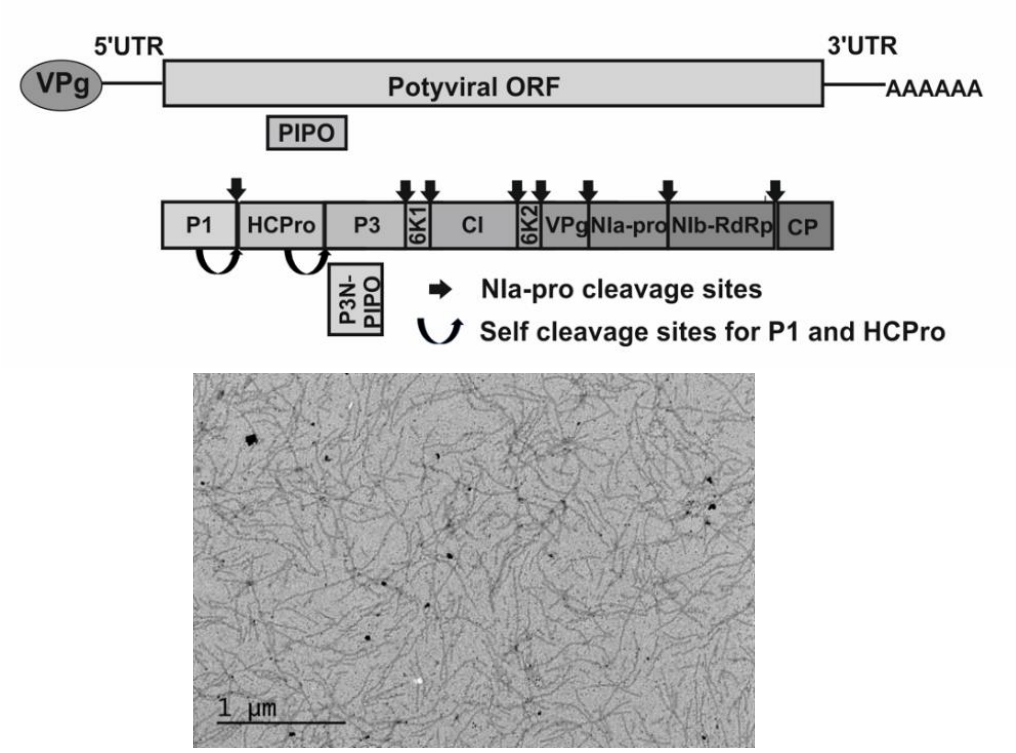


Figure 1: Schematic representation showing the Potato Virus A (PVA) genome organisation. The PVA genome consists of positive single-stranded (+ssRNA), with a covalently linked viral protein genome-linked (VPg) at the 5' end and a polyadenylated tail at the 3' end. The PVA genome has one long open reading frame (ORF) and one short ORF, called pretty interesting potyvirus ORF (PIPO). PVA RNA is directly translated, producing one long and one short polyprotein. The long polyprotein is subsequently cleaved into 10 individual proteins. The short polyprotein is cleaved into three individual proteins, namely P1, helper component proteinase (HCPro), and P3N-PIPO. Cleavage sites are indicated by

arrows. In the bottom image, PVA particles from infected samples collected at 3 days post-infection are shown under electron microscopy.

1.1.3 Infection cycle of potyviruses

The infection cycle of potyviruses includes virus entry, translation, replication, antiviral defence/counter defence, encapsidation, and movement. These stages partially overlap and consist of several sub-stages that are connected by a complex regulatory interaction network. Under natural conditions, potyviruses are transmitted to host cells by aphids, in a non-persistent manner. Upon cellular entry, the virus uncoats the virion and releases viral RNA (vRNA) into the host cytoplasm. The first round of translation occurs from the released vRNA, to produce a set of the proteins that are necessary for viral replication. Infection with an adequate quantity of vRNA is crucial during this step to ensure that sufficient vRNA can be translated before the RNA is degraded. Potyviral RNA serves as a template for both replication and translation. These two processes are mutually exclusive but may be closely associated (Cotton et al., 2009; Grangeon et al., 2010; Hafrén et al., 2010). The translation of the full large ORF is required for the replication of potyvirus, which ensures that only those vRNAs that contain the full ORF will be replicated (Mahajan et al., 1996). Potyvirus replicates in an endoplasmic reticulum (ER)-derived, membrane-bound complex that consists of both host and viral proteins, known as the virus replication complex (VRC). The VRC is formed by the cylindrical inclusion (CI) helicase, 6K2, VPg proteinase (NIa), and the RdRP (NIb) (Schaad et al., 1997a). NIb catalyses the uridylation of VPg, which enables VPg to act as a primer for vRNA replication (Anindya et al., 2005; Puustinen and Mäkinen, 2004). Potyviral translation occurs first at ER-derived membranes. At the ER exit sites, the viral protein 6K2 remodels the ER membrane to recruit vRNA and replication-associated proteins, resulting in the formation of the VRC (Cotton et al., 2009; den Boon and Ahlquist, 2010; Grangeon et al., 2012; Laliberté and Sanfaçon, 2010; Miller and Krijnse-Locker, 2008; Schaad et al., 1997a; Verchot, 2011; Wei et al., 2010a; Wei and Wang, 2008). The 6K2-containing VRCs exit the ER site and fuse with chloroplast membranes. Active viral replication begins in the VRC-fused chloroplast membrane (Wei et al., 2010a). VRCs are often associated with host proteins involved in translation, especially eIF4E, eukaryotic elongation factor 1A (eEF1A), RNA helicase-like protein RH8, poly(A) binding protein (PABP), and heat shock protein 70 (HSP70) (Beauchemin et al., 2007; Beauchemin and Laliberté, 2007; Cotton et al., 2009; Dufresne et al., 2008; Huang et al., 2010; Thivierge et al., 2008), and viral proteins, such as HCPro, P3, CI, and NIa. These

viral proteins have also been proposed to be involved in viral replication (Ala-Poikela et al., 2011; Cui et al., 2010; Hong and Hunt, 1996; Li et al., 1997; Merits et al., 1999; Merits et al., 2002). However, a recent mass spectrometry study examining the PVA 6K2-bound VRC revealed that eight of eleven PVA proteins were abundantly expressed in PVA VRCs. Among the remaining three viral proteins, P3N-PIPO and P1 could not be detected, and CP was detected at a low level (Lõhmus et al., 2016). Potyviral replication occurs via the synthesis of intermediate minus (−)-strands, which are then used as templates to produce millions of copies of plus-strands. Replicated vRNA can then proceed to cell-to-cell movement or continue the translation process. A crucial step between these procedures is the protection of vRNA from host-mediated silencing. vRNA can trigger host silencing machinery and eventually undergo degradation (reviewed by (Carrington et al., 2001). Recently, a potyvirus-induced granule (PG) structure was discovered in PVA infected cells (Hafrén et al., 2015). PGs have been proposed to act as a compartment for protecting vRNA from degradation. PGs are intermediate structures between PVA replication and translation and are closely associated with VRCs (Hafrén et al., 2015). PGs are composed of vRNA and a variety of host and viral proteins, such as HCPro, which is the primary initiator of PG formation, VARICOSE (VCS), ARGONAUTE 1 (AGO1), the eIF4E isoform (eIF(iso)4E), P0, and oligo-uridylate binding protein 1 (UBP1). The emerging picture suggests that HCPro induces PGs to store vRNA and protect them from host-mediated silencing until VPg can translate them. The turnip mosaic virus (TuMV) VPg was recently found to resist the autophagy-mediated degradation of TuMV HCPro-induced RNA granules (Hafrén et al., 2018).

Potyviruses modify plasmodesmata (PD) to support cell-to-cell movements. Potyviral proteins, such as P3N-PIPO, CI, and CP, participate in cell-to-cell movement (Carrington et al., 1998; Dolja et al., 1995; Dolja et al., 1994; Wei et al., 2010b; Wen and Hajimorad, 2010). These proteins, along with vRNA, are localised in PD (Roberts et al., 1998; Rodríguez-Cerezo et al., 1997), where CI forms conical structures and P3N-PIPO acts as an anchor linking the CI to the PDs (Wei et al., 2010b). Potyvirus RNA can move cell-to-cell, either as a vesicle-bound structure (Grangeon et al., 2013) or as a virion. For the tobacco etch virus (TEV), the CP and vRNA assembly are crucial for viral movement (Dolja et al., 1994). The phosphorylation of PVA CP affects its RNA-binding function (Ivanov et al., 2001), replication (Lõhmus et al., 2017), and viral movement through infected plants (Ivanov et al., 2003). This finding suggests that the capacity of CP

to assemble vRNA is an essential factor for the cell-to-cell movement of potyviruses. Recent studies (Gallo et al., 2018) have shown that the CP assembly of vRNA for virion formation is coupled with replication. These findings indicate that the lack of replication can affect virion formation and, consequently, affect cell-to-cell movement. De (2019) (De, 2019) described a replicating virus, PVA^{WD} (described in detail in the Materials and Methods and Results sections), that failed to produce particles and was not capable of systemic movement. This finding indicated that long-distance movements appear to require particle formation, whereas cell-to-cell movements can also occur via VRCs. However, additional evidence remains necessary to establish this phenomenon.

vRNA can undergo further rounds of translation. Potyviral RNA carries VPg, which is covalently-bound to the 5′ end of vRNA, instead of a 5′ cap structure (Adams et al., 2005). The 5' untranslated region (UTR) contains an internal ribosomal entry site (IRES)-element for the initiation of cap-independent translation. Potyviruses have a larger VPg and a shorter 5' UTR, unlike the UTRs of other *Picornaviridae* family members. The potyviral IRES element lacks a strong structure and does not contain any AUG triplets (Miras et al., 2017). VPg contributes directly to translation efficiency and is necessary to initiate potyviral translation. VPg acts as a cap-like structure and recruits the host translational machinery to form a translational initiation complex (Khan et al., 2008; Miyoshi et al., 2008; Miyoshi et al., 2006). The 3'poly(A)-binding protein (PABP), interacts with the 5' translational complex, which enhances the stability of the complex and initiates translation (Khan and Goss, 2012; Khan et al., 2008). After translation, P1 and HCPro are separated from the polyprotein by a self-cleavage mechanism, and the remaining proteins are separated by NIa-pro. Following translation, vRNA replication continues to use the newly formed vRNA molecules as templates or the vRNA molecules are used for particle formation and transmission. Particle formation begins when sufficient quantities of CP have accumulated in the cell. *Trans* CP binds to the translating CP molecule associated with vRNA. A chain of CP molecules encapsidates vRNA particles through CP-CP interactions, resulting in virion formation (Besong-Ndika et al., 2015). HCPro plays a key role in stabilising the particles (Valli et al., 2014). The potyviral virion contains VPg (Oruetxebarria et al., 2001; Puustinen and Mäkinen, 2004; Torrance et al., 2006), HCPro (Torrance et al., 2006), CI (Gabrenaite-Verkhovskaya et al., 2008), and host eIF4E (Tavert-Roudet et al., 2017) in the 5' tip structure. eIF4E has been proposed to protect vRNA from degradation during disassembly,

ensuring translation. A schematic picture of the PVA infection cycle is presented in Figure 2.

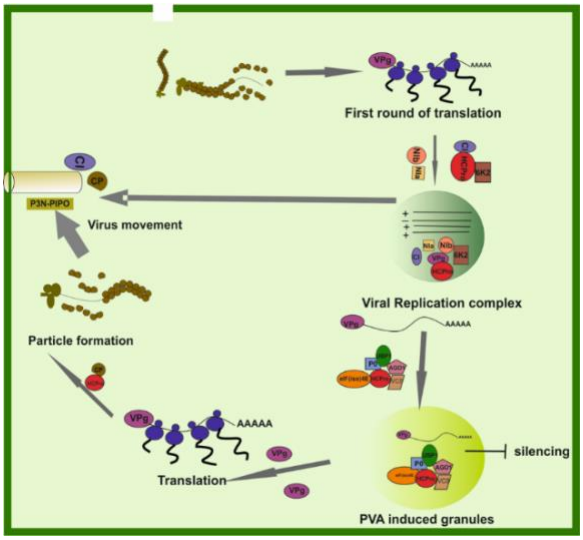


Figure 2: Schematic representation of potyviral infection in a single cell. Potyviruses enter the cell through aphid transmission or mechanical inoculation. The virus uncoats inside the cell and releases viral RNA (vRNA) into the cytoplasm. An initial round of translation occurs to produce the viral proteins necessary for the formation of viral replication complexes (VRCs). Upon replication, vRNAs move to potyvirus-induced granules (PGs), which serve to suppress RNA silencing mechanisms. vRNA is transferred to polysomes for the translation of viral proteins. Translation is followed by encapsidation and movement or re-entry into a new round of replication. Alternatively, some evidence suggests that vRNAs can move to new cells within membrane-bound vesicles.

1.1.4 Virulence determinants in potyviruses

Plants have evolved various sophisticated strategies to defend themselves against viral infection. The primary antiviral defence response is RNA silencing. RNA silencing or RNA interference (RNAi) is a widely used defence mechanism against viruses (Ding, 2010; Pumplin and Voinnet, 2013). RNA silencing is counter- defended by virus-encoded suppressors of RNA silencing (VSRs) (Garcia-Ruiz et al., 2016; Zhao et al., 2016). The other well-studied virus- and host-specific resistance mechanism is known as gene-for-gene resistance. Plant resistance gene (R-gene)-encoded proteins recognise viral avirulence factors (Avr), either through direct interactions or indirectly via modifications, as

proposed by the guard or decoy models (Jones and Dangl, 2006; Moffett, 2009). R genes can confer either dominantly or recessively inherited resistance. Dominant R genes confer resistance primarily against bacterial and fungal pathogens but can also confer resistance against viruses (Moffett, 2009; Padmanabhan and Dinesh-Kumar, 2014). Recessive resistance (r) genes are more commonly associated with resistance against viruses (Truniger and Aranda, 2009; Wang and Krishnaswamy, 2012). Approximately half of the alleles known to convey viral resistance in plants are recessive (Kang et al., 2005b). Plants also use other cellular mechanisms, including autophagy (Hafrén and Hofius, 2017; Haxim et al., 2017; Liu et al., 2018), RNA methylation (Martínez-Pérez et al., 2017) and ubiquitination (Alcaide-Loridan and Jupin, 2012), to combat viral attacks. The present study focuses on the eIF4E/(iso)4E-mediated recessive resistance against potyviruses, which is described in detail below.

1.1.4.1 Virulence by overcoming eIF4E/(iso)4E-mediated recessive resistance

Two hypotheses have been proposed as the basis for recessive resistance. The first hypothesis is based on the failure of direct or indirect molecular interactions between viral and host factors, which are required for the virus to propagate in host cells (Albar et al., 2006; Pyott et al., 2016). The second hypothesis is based on an active mechanism, in which resistance genes are autoactivated by the recognition of virus-encoded molecules to initiate plant defence responses (Diaz-Pendon et al., 2004; Truniger and Aranda, 2009). A number of recessively inherited resistance sources have been identified against viruses in the *Potyviridae* family (Hampton and Provvidenti, 1992). The potyviral VPg interaction with eIF4E is vital for viral multiplication (Léonard et al., 2000; Wittmann et al., 1997). The eIF4E gene was the first-identified, natural, recessive gene, which was found to confer resistance against potato virus Y (PVY), TEV (Ruffel et al., 2002), and lettuce mosaic virus (LMV) (Duprat et al., 2002). The map-based cloning of *Arabidopsis thaliana* mutants, which exhibited the loss-of-susceptibility against TEV and turnip mosaic virus (TuMV), resulted in the identification of eIF(iso)4E as being responsible for susceptibility loss (Lellis et al., 2002). Subsequent studies revealed that several recessive resistance genes against potyviruses and other plant viruses belonged to the eIF family, including eIF2Bβ, eIF4E, eIF4G, and their isoforms (Shopan et al., 2017; Truniger and Aranda, 2009). eIF-mediated resistance against many potyviruses has been studied extensively in a large number of cultivated plants (Bruun-Rasmussen et

al., 2007; Gallois et al., 2010; Hashimoto et al., 2016; Hwang et al., 2009; Jenner et al., 2010; Kanyuka et al., 2005; Kühne et al., 2003; Léonard et al., 2000; Ling et al., 2009; Wang and Krishnaswamy, 2012). Altogether, 19 recessive and 2 dominant genes, have been identified, thus far, as natural resistance genes encoding members of the eIF family (Shopan et al., 2020; Wang and Krishnaswamy, 2012). These genes include: *mo1* in lettuce (*Lactuca sativa*); *nsv* in melon (*Cucumis melo*); *pot-1* in tomato (*Solanum lycopersicum*); pvr^1, pvr^2, and pvr^6 in peppers (*Capsicum annuum* and *C. chinense*); *rym4, rym5,* and *rym6* in barley (*Hordeum vulgare*); *sbm1*, *wlv,* and *cyv2* in pea (*Pisum sativum*); *retr01* and *retr02*, in Chinese cabbage (*Brassica rapa*) (Qian et al., 2013; Rusholme et al., 2007), and *retr03* in *Brassica juncea* (Shopan et al., 2017). Importantly, *restricted TEV movement 3* (*RTM3*), which acts as a dominant TuMV resistance gene, encodes an eIF3b subunit in *Arabidopsis thaliana* (Rubio et al., 2019).

1.1.4.2 Role of eIF4E/(iso)4E in infected host cells

Host cellular mRNAs contain a 5' 7-methylguanosine-(5′) triphospho (5′) guanosine (m7GpppG) structure, which referred to as the 5'cap, an ORF, and a poly(A) tail at the 3′-end (Kozak, 1999; Nicholson and White, 2011). In eukaryotic mRNAs, the 5′-cap structure recognises and recruits eIF4E, which is necessary for the efficient recruitment of translation initiation factors (Amrani et al., 2008; Wang et al., 2009). The eukaryotic translation initiation factor F (eIF4F) is a heterodimer of eIF4E and a core scaffolding protein, eIF4G, to which the other factors bind (Miller et al., 2007; Nicholson and White, 2011; Wang et al., 2009). eIF4A, an ATPase/RNA helicase, interacts with eIF4F but is not a component of the eIF4F heterodimer (Gallie and Browning, 2001). The large scaffolding protein eIF4G binds to both eIF4E and PABP from the 3' UTR, promoting the circularisation of mRNA. For translation initiation, eIF4E serves to recruit eIF4G to the capped mRNA. This complex is responsible for 5′ cap recognition, mRNA unwinding, and the recruitment of the 40S ribosomal subunit (Gingras et al., 1999; Kawaguchi and Bailey-Serres, 2002; Nicaise et al., 2007; Perez et al., 2012; Raught et al., 2004). In addition, eIF4G recruits the 43S ternary ribosomal complexes via interactions with eIF3.

Viruses use host cellular translation factors to accomplish viral protein synthesis. (+)-strand vRNAs differ from typical cellular mRNAs in various aspects. Depending on the virus group, some vRNA molecules feature a 5' cap or a poly(A) tail, whereas others do not. Some vRNA molecules feature highly

structured 5' UTRs that contain stem-loops and pseudoknots, which facilitate infection, and IRES, which enable translation at downstream ORFs (Sanfaçon, 2015). Thus, viruses have developed several alternative mechanisms through which translate vRNAs. These non-canonical translational mechanisms have been reviewed in detail by many authors (Au and Jan, 2014; Chujo et al., 2015; Firth and Brierley, 2012; Miller et al., 2015; Miras et al., 2017; Simon and Miller, 2013; Thompson, 2012; Zhang et al., 2015). Viruses can also control the translational machinery of their hosts (Echevarría-Zomeño et al., 2013; Jiang and Laliberté, 2011; Walsh et al., 2013). Thus, a key factor necessary for successful infection is the ability of the virus to use the host translation machinery efficiently. In the case of (+)-stranded RNA viruses, the translation initiation of vRNA is necessary to avoid vRNA degradation upon entry into the host cell (Miras et al., 2017; Nieto et al., 2011; Truniger et al., 2008).

VPg covalently attaches to the 5′ end of potyviral RNAs, in place of the cap typically found on cellular mRNAs. VPg contributes directly to translational efficiency by interacting with translational initiation factors, both *in vitro* and *in planta* (Beauchemin et al., 2007; Khan et al., 2008; Miyoshi et al., 2008), which has been supported by several studies. VPg competes with the m7G cap and binds with its preferential form of eIF4E more efficiently than the m7G cap (Khan et al., 2006; Miyoshi et al., 2006). In wheat germ extract that was depleted of the cap-binding partners eIF(iso)4F and eIF(iso)4E, TEV VPg interacted with ectopically expressed eIF(iso)4E to enhance the translation of uncapped IRES-containing TEV RNA. Disrupting the VPg and eIF(iso)4E interaction abolished IRES-mediated translation (Khan et al., 2008). TuMV VPg forms a stable complex with eIF(iso)4E and PABP, both *in vitro* and *in vivo* (Khan and Goss, 2012; Khan et al., 2008; Léonard et al., 2004). In PVA, the ectopic expression of VPg enhances PVA translation *in planta* (Eskelin et al., 2011). In addition to translation, VPg provides stability to vRNA and protects vRNA from degradation (Eskelin et al., 2011). Evidence has suggested that VPg increased the amount of vRNA found in leaves that were infected with either wild-type or non-replicating PVA (Eskelin et al., 2011). eIF4E/eIF(iso)4E participates in the transportation of potyviral RNA as part of a ribonucleoprotein (RNP) complex, through interactions with cellular microtubules (Contreras-Paredes et al., 2013; Lellis et al., 2002; Okade et al., 2009). eIF4E/eIF(iso)4E and other eIFs, such as eEF1A, and PABP are components of the virus translation/replication complex (Beauchemin et al., 2007; Thivierge et al., 2008; Wei et al., 2010a). Recently, eIF4E was found to be associated with VPg at the 5' end of the virions, which is

likely to increase the availability of eIF4E for translation, immediately after the virions enter new cells (Tavert-Roudet et al., 2017). A recent study by Ala-Poikela et al. (2019), indicated that the disruption of the VPg–eIF(iso)4E interaction by an alanine mutation in the YTDIRL motif might affect the RNA silencing suppression capacity and, therefore, compromise the infection efficiency.

1.1.4.3 Molecular details of VPg–eIF4E/(iso)4E interactions

The conditions for a successful VPg and eIF4E/(iso)4E interaction are fairly variable among potyviruses and are specific to a particular host-virus pathosystem. Variations in the interactions across the potyvirus families are typically generated through two primary mechanisms: (1) the differential usage of host eIFs among potyviruses; and (2) specific molecular changes or point mutations that occur in both VPg and eIFs, which serve as both attack and defence strategies. VPg acts as a significant virulence determinant for many potyviruses (Borgstrøm and Johansen, 2001; Gallois et al., 2010; Huang et al., 2010; Kühne et al., 2003; Léonard et al., 2000; Moury et al., 2004; Perez et al., 2012; Rajamäki and Valkonen, 1999, 2002).

A given viral VPg may prefer to interact with eIF4E, eIF(iso)4E, or both in a specific host species or it can recruit eIFs in a selective and coordinated manner (Nicaise et al., 2007; Sato et al., 2005). Some potyvirus VPgs may show similar affinities to both host and non-host eIFs, either naturally (Okade et al., 2009) or conditionally, which is exemplified by TuMV, which can use both eIF4E and eIF(iso)4E of *Brassica rapa* to accomplish replication in an *Arabidopsis* eIF(iso)4E knockout (KO) mutant (Jenner et al., 2010). The preferred interaction partners of a given viral VPg also depends on the host, as exemplified by LMV, which interacts with eIF4E when infecting lettuce but preferentially interacts with eIF(iso)4E when in *Arabidopsis* (Duprat et al., 2002; Nicaise et al., 2003; Ruffel et al., 2005). Additionally, eIFs and their isoforms can preferentially bind with specific mRNAs/vRNAs, adding yet another level of complexity to the infection context (Martínez-Silva et al., 2012; Mayberry et al., 2011). The binding sites in both proteins are highly polymorphic. The resistance and susceptibility-specific mutations in eIF4E/(iso)4E and VPg, respectively, have evolved through the positive selection of a few non-synonymous point mutations among many mutations. These mutations are typically not conserved across the potyvirus species (Gao et al., 2004; Kang et al., 2005b; Moury et al., 2014; Nicaise et al.,

2003; Ruffel et al., 2002; Ruffel et al., 2005; Wang and Krishnaswamy, 2012; Yeam et al., 2007). The amino acids required eIF4E to interact with VPg are clustered near eIF4E's cap-binding pocket, inside the pocket, on the outer surface of the fingers forming the pocket (Kang et al., 2005a; Nicaise et al., 2003; Ruffel et al., 2005; Stein et al., 2005), and on the surface 90° from the cap-binding pocket (Monzingo et al., 2007; Robaglia and Caranta, 2006). Changes in a single or a few amino acids can introduce resistance to potyviruses (Charron et al., 2008; Robaglia and Caranta, 2006). This conferred resistance to potyviruses is so specific that even within a single plant, different eIF4E alleles can contribute to resistance against the same or different virus strains (Charron et al., 2008; Nicaise et al., 2003). The inhibition of the VPg–eIF4E interaction due to point mutations in eIF4E does not affect the cap-binding functions of eIF4E, facilitating normal cellular function (German-Retana et al., 2008; Ibiza et al., 2010; Piron et al., 2010).

The majority of VPg mutations that affect resistance conferred by recessive eIF4E/eIF(iso)4E mutations can be found in the central or C-terminal domain of VPg. The virulence determinants have been mapped in most resistance-breaking potyviral isolates. The central and C-terminal domains of the VPg have been implicated in the eIF4E interaction and viral infection (Borgstrøm and Johansen, 2001; Bruun-Rasmussen et al., 2007; Charron et al., 2008; Duprat et al., 2002; Gallois et al., 2010; Grzela et al., 2006; Moury et al., 2004; Perez et al., 2012; Rajamäki and Valkonen, 1999, 2002; Roudet-Tavert et al., 2007; Wittmann et al., 1997; Yambao et al., 2003). Several studies have shown that a direct interaction between VPg and eIF4E/(iso)4E is necessary for viral susceptibility. The results from a yeast two-hybrid system and an enzyme-linked immunosorbent assay (ELISA) showed the direct interaction between TuMV VPg and the *Arabidopsis thaliana* eIF(iso)4E, for the first time (Wittmann et al., 1997). The formation of a complex between these two proteins correlated with TuMV infectivity (Léonard et al., 2000). Direct interactions between TEV VPg and eIF4E from a susceptible tomato variety and between LMV VPg and eIF4E from lettuce have also been demonstrated by yeast two-hybrid and ELISA assays (Roudet-Tavert et al., 2007; Schaad et al., 2000). Glutathione-S-transferase (GST) pull-down and yeast two-hybrid assays have revealed a strong interaction between TEV VPg and eIF4E from susceptible pepper varieties. In contrast, eIF4E resistant varieties (pvr^1, pvr^2, and pvr^6) failed to bind TEV VPg (Kang et al., 2005a). Viruses are highly adaptable and are capable of overcoming resistance by undergoing reversion, and the introduction of other compensatory mutations in VPg can also restore

interactions. Thus, resistance-breaking isolates often feature restored interactions with the host eIF4E/(iso)4E. For example, the resistance breaking PVY VPg isolates can interact with the host eIF4E and overcome the resistance of the pvr^2 allele (Moury et al., 2004), which suggests the existence of continuous co-evolutionary processes between hosts and pathogens. Additionally, resistance-breaking can also be achieved through a *de novo* interaction between hosts and pathogens. For example, a single amino acid change in TuMV VPg is sufficient to overcome the *A. thaliana* eIF(iso)4E resistance allele (Charron et al., 2008).

1.1.5 CP regulation and particle formation in potyvirus

In addition to VPg, CP is also involved in several regulatory functions and host interactions during potyvirus infection (Ivanov and Mäkinen, 2012). The CP cistron is located at the extreme 3' side of the viral genome and is the last cistron to be translated from the long PVA ORF. Approximately 2,000 CP subunits are required to form a helical arrangement around the potyviral RNA, which generates a flexuous rod-shaped, 680–900-nm-long, and 11–13-nm-wide virion (López-Moya et al., 2009). Based on the secondary structure proposed by Baratova et al., (2001) (Baratova et al., 2001), the PVA CP structure can be divided into three regions, the N-terminal region, the central region, and the C-terminal region. The central, highly conserved region consists of five alpha-helices, which form the core structure of the virus particle (Dolja et al., 1994; Varrelmann and Maiss, 2000; Voloudakis et al., 2004). The N-terminal region is highly unstructured, consisting of 4 beta-strands. The C-terminal domain folds into two alpha-helices and three beta-strands (Baratova et al., 2001). Both the N- and C-termini are exposed on the surface of the virion (Allison et al., 1985; Shukla et al., 1988) and are involved in CP-CP inter-subunit interactions that are necessary for the initiation of virus assembly (Anindya and Savithri, 2003; Kang et al., 2006; Seo et al., 2013). The N-terminal region of CP is highly variable among potyviruses and has been predicted to be disordered. The predicted disorder in this region is conserved, which indicates that the structural flexibility of this region likely has biological relevance (Charon et al., 2016; Ksenofontov et al., 2013; Peng et al., 2015; Rybicki and Shukla, 1992). PVA CP undergoes phosphorylation, which reduces its affinity for vRNA and is necessary to avoid premature particle formation (Ivanov et al., 2003; Ivanov et al., 2001). Plum-pox virus (PPV) CP is phosphorylated and O-Glc-Nacylated. O-Glc-Nacylation enhances viral infection efficiency and CP stability (Chen et al., 2005; Martínez-Turiño et al., 2018; Pérez et al., 2013).

CP is involved in multiple functions, including encapsidation, cell-to-cell movement, aphid transmission, and viral gene regulation (Ivanov and Mäkinen, 2012; Revers and García, 2015). For many (+)-sense RNA viruses, CP modulates gene expression in a concentration-dependent manner (Boni et al., 2005; Yi et al., 2009a). A higher CP concentration represses RNA accumulation, whereas lower levels stimulate RNA accumulation and translation. Thus, tight control over CP production is necessary, depending on the stage of virus infection. CP expression from subgenomic RNAs (sgRNAs) can represent one method used to regulate CP expression levels, allowing for CP to be expressed in significant quantities only after successful viral replication (Annamalai and Rao, 2006). Potyviruses lack sgRNA and are known to produce equimolar amounts of all proteins from the CP-containing polyprotein. Several studies have revealed that potyviruses also control CP accumulation during active gene expression by using host cellular systems to degrade CP, which involves a host kinase, two chaperones, and the proteasomal degradation system (Besong-Ndika et al., 2015; Hafrén et al., 2010; Ivanov et al., 2003; Lõhmus et al., 2017). An intriguing question is how a potyvirus produces the large number of CPs necessary to generate an adequate number of particles necessary for aphid transmission and systemic movement. For example, the particles shown in Figure 1 (bottom image) were generated by a PVA infection in inoculated *N. benthamiana* leaves within the first 3 days of infection. An alternative mechanism for CP production may be required to generate sufficient quantities of CP that are necessary to produce a large number of potyvirus particles.

HCPro is multi-functional and interacts with multiple host and viral partners (as reviewed in (Revers and García, 2015; Valli et al., 2018). HCPro was first recognised as a helper component that was required for the aphid transmission of viral particles (Atreya et al., 1992; Thornbury et al., 1985). Later, HCPro was recognised as the major protein acting as a host silencing suppressor (Anandalakshmi et al., 1998; Kasschau and Carrington, 1998). HCPro is involved in several other functions, including polyprotein processing (Carrington et al., 1989), virion formation (Valli et al., 2014), and systemic movement (Cronin et al., 1995; Kasschau et al., 1997). HCPro and CP interact *in vitro* and *in vivo* (Roudet-Tavert et al., 2002). Valli et al. (2014) showed that a domain of HCPro that is not involved silencing suppression contributed to enhancing the yield of particles in PPV, which suggested that HCPro could stabilise the CP proteins required for particle formation. Furthermore, Gallo et al. (2018) proposed that

particle formation required replication-competent PPV RNA, as replication-deficient PPV could not stabilise particles. In light of these previous studies, stable particle formation in potyviruses appears to involve many viral and host proteins. Thus far, the viral proteins VPg, HCPro, and CI and the host protein eIF4E have been associated with a tip structure at the other end of potyviral particles (Gabrenaite-Verkhovskaya et al., 2008; Puustinen et al., 2002; Tavert-Roudet et al., 2017; Torrance et al., 2006). Whether these proteins are required to initiate CP assembly or are involved in particle stability remains unknown. The association between replication and virion formation may be necessary to ensure the infectivity of viral particles. How viruses recruit these proteins into the tip of the particle remains an open question. One possibility is that potyviral RNA initiates particle formation directly from the polysome, as has been previously proposed (Besong-Ndika et al., 2015).

2. AIMS OF THE STUDY

1. To understand the molecular mechanisms underlying the interaction between VPg and eIF(iso)4E during PVA infection and to analyse the biological functionality of VPg^{mut}, which lacks the eIF(iso)4E binding site, compared with wild-type VPg.

2. To understand how potyvirus produces large amounts of CP from the polyprotein, which is necessary for adequate particle formation.

3. To understand the role of HCPro during the stabilisation of CP and virion formation.

3. MATERIALS AND METHODS

3.1 Plants

Nicotiana benthamiana plants (NCBI: taxid4100) were used in all the experiments. Plants were grown at 22 ºC under 16 h light / 8 h dark photoperiod in environmentally controlled greenhouses. A Draft genome sequence is present in Sol Genomics Network (https://solgenomics.net/;(Bombarely et al., 2012).

3.2 Viruses and viral constructs

PVA strain B11 (GenBank accession number AJ296311) was used in all the experiments. Specific mutations were made and listed in Table 3

3.3 Other methods and list of constructs

All the methods used in this study are described in the publications as indicated in Table 2. A number of different viral constructs, as well as various expression and silencing constructs used in this study are presented in Table 3.

Table 2. Methods used for this study along with the articles containing experimental details

Methods	Publication
Agrobacterium infiltration	**I, II, III**
Confocal microscopy	**II, III**
Dual luciferase assay	**I, II, III**
Electron microscopy	**II,**
Epifluorescence microscopy	**II**
Immunocapture-RT-qPCR (IC-RT-qPCR)	**II, III**
Molecular cloning	**I, II**
Quantitative RT-PCR (qRT-PCR)	**I, II, III**
Enzyme Linked Immunosorbent Assay (ELISA)	**I, II**
Western blot analysis	**I, II, III**

Table 3. Molecular constructs used in this study along with brief description

Binary vectors carrying PVA infectious cDNA				
Construct name	**Gene Cassette**	**Vector**	**Description**	**Ref**
PVA^{WT}: RLUCCP	35S-PVAWT $RLUC^{int}$-nos	pRD400	Full length wild type PVA icDNA tagged with RLUC present in between NIb and CP cistron	(Kelloniemi et al., 2008) **I, II, III**
PVA^{WT}: RLUCH	35S-PVA^{WT}:$RLUC^{int}$-nos	pRD400	Full length wild type PVA icDNA tagged with RLUC present in between P1 and HCPro cistron	(Kelloniemi et al., 2008) **I, III**
$PVA^{\Delta GDD}$: RLUCCP	35S-$PVA^{\Delta GDD}$: $RLUC^{int}$-nos	pRD400	Full length replication deficient PVA icDNA tagged with RLUC present in between NIb and CP cistron	(Eskelin et al., 2010) **I, II, III**
$PVA^{\Delta GDD}$: RLUCH	35S-$PVA^{\Delta GDD}$:$RLUC^{int}$-nos	pRD400	Full length wild type PVA icDNA tagged with RLUC present in between P1 and HCPro cistron	**I**

PVA^{VPgmut}	35S-PVAVPg mut :$RLUC^{int}$-nos	pRD400	Full length wild type PVA icDNA, having 2 Alanine substitution in eIF(iso)4E binding site, tagged with RLUC present in between NIb and CP cistron	**II**
PVA^{CPmut}	35S-PVA-CPmut $RLUC^{int}$-nos	pRD400	Full length wild type PVA icDNA, having substitution mutations R159D and Q160Vin eIF(iso)4E binding site, tagged with RLUC present in between NIb and CP cistron	(Eskelin et al., 2010) **II, III**
PVA^{ADA}	35S-PVA^{ADA} $RLUC^{int}$-nos	pRD400	Full length wild type PVA icDNA, having substitution mutations with 242 TTS to ADA in CP tagged with RLUC present in between NIb and CP cistron	(Lõhmus et al., 2017) **III**
PVA^{AAA}	35S-PVA^{AAA} $RLUC^{int}$-nos	pRD400	Full length wild type PVA icDNA, having substitution mutations with 242 TTS to AAA in CP tagged with RLUC present in between NIb and CP cistron	(Ivanov et al., 2003) **III**

PVA^{WD}	35S-PVA^{WD}:RLUC^{int}-nos	pRD400	RLUC-tagged full-length infectious cDNA clone of PVA with mutation in the site encoding for WD domain interacting motif of HCPro	(De et al., 2020)**III**
PVA$^{\Delta HCPro}$	35SPVA$^{\Delta HCPro}$:RLUC^{int}-nos	pRD400	PVA lacking HCPro tagged with RLUC	(Hafrén et al., 2015) **III**
PVA$^{\Delta GDD\ B\text{-}box}$	35S- PVA$^{\Delta GDD-\ B\text{-}box}$ - nos	pRD400	Full length replication deficient PVA icDNA tagged with lambda phage B-box element at 3' side	(Hafrén et al., 2015) **II**

Protein expression constructs

Construct name	**Gene Cassette**	**Vector**	**Description**	**Ref**
VPg	35S-VPg-nos	pRD400	Plasmid expressing PVA VPg	**(**Eskelin et al., 2011) **I, II**
VPg^{mut}	35S-VPg^{mut}-nos	pRD400	Plasmid expressing PVA VPg^{mut}	**II**
P0	35S-P0-nos	pRD400	Plasmid expressing *N. benthamiana* P0	(Hafrén et al., 2013) **I**
P0^{YFP}	35S-P0^{YFP}-nos	pRD400	Plasmid expressing *N. benthamiana* P0 tagged with YFP	(Hafrén et al., 2015) **II**
FLUC	35S-FLUC-nos	pRD400	Plasmid expressing intron spliced FLUC	(Eskelin et al., 2010**)** **I, II, III**

GUS	35S-GUS-nos	pRD400	Plasmid expressing uidA gene encoding β-glucuronidase (GUS)	(Eskelin et al., 2010) **I, II, III**
eIF(iso)4E ^{RFP}	35S-eIF(iso)4E RFP-nos	pSITEII6 C1	Plasmid expressing *N. benthamina* eIF(iso)4E fused with RFP	(Hafrén et al., 2015) **II**
λN22^{RFP}	35S- λN22 RFP-nos	pSITEII6 C1	Plasmid expressing λN22 fused with RFP	(Hafrén et al., 2015) **II**
HCPro	35S-HCPro-nos	pRD400	Plasmid expressing PVA HCPro	(Hafrén et al., 2015) **I,III**
HCPro^{4Ebd}	35S-HCPro$_{4Ebd}$-RFP-nos	pSITEII-6C1	Plasmid expressing eIF4E binding deficient mutant of HCPro fused to RFP	(Hafrén et al., 2015), **III**
HCPro^{SD}	35S-HCPro$^{SDM-RFP}$-nos	pSITEII-6C1	Plasmid expressing silencing suppression deficient mutant of HCPro fused to RFP	(Hafrén et al., 2015), **III**
HCPro^{WD}	35S-HCPro^{WD}-nos	pRD400	Plasmid expressing PVA HCPro^{WD}	(De et al., 2020)**III**
2b	35S-2b-nos	pRD400	Plasmid expressing silencing suppressor 2b	(Hafrén et al., 2015), **III**
CP	35S-CP-nos	pRD400	Plasmid expressing RLUC	(Lõhmus et al., 2017),**III**

4. RESULTS AND DISCUSSION

4.1 Article II: Insights into the VPg–eIF(iso)4E interaction during PVA Infection

The functional interaction between VPg and eIF4E/(iso)4E is an important virulence determinant that supports potyvirus infection (Borgstrøm and Johansen, 2001; Gallois et al., 2010; Léonard et al., 2004; Moury et al., 2004; Perez et al., 2012; Rajamäki and Valkonen, 1999, 2002) and has been confirmed by studying many different model plants and crop plants, as previously reviewed comprehensively (Robaglia and Caranta, 2006; Truniger and Aranda, 2009; Wang and Krishnaswamy, 2012). This interaction is intricate and has developed through various co-evolutionary processes between the host and the viral species (Charron et al., 2008; Moury et al., 2014; Nicaise, 2014). However, how the interaction exerts its effect remains poorly understood. This study investigated the VPg and eIF(iso)4E interaction during PVA infection by mutating the eIF(iso)4E binding site in VPg.

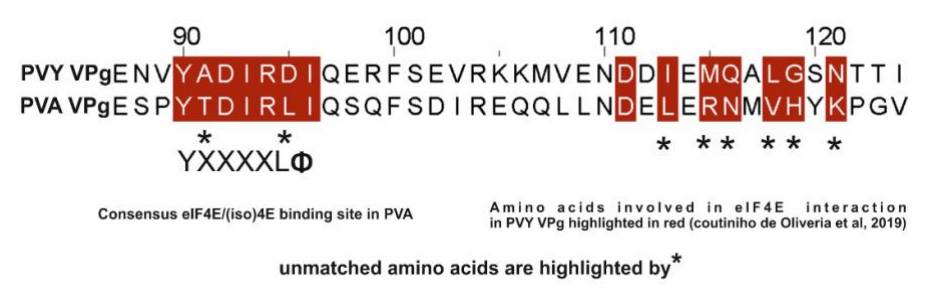
4.1.1 A Consensus eIF(iso)4E Binding site was identified in PVA VPg

EIF4E-binding proteins contain a conserved Tyr-X-X-X-X-Leu-phi (YXXXXLΦ) motif, through which they bind to eIF4E (Marcotrigiano et al., 1999; Rhoads, 2009). In this sequence, X represents any amino acid, and phi represents any hydrophobic amino acid. The PVA proteins VPg and HCPro contain a similar recognition motif, YTDIRLI, which interacts with the host eIF(iso)4E protein (Ala-Poikela et al., 2011; Ala-Poikela et al., 2019). The substitution of the tyrosine and the leucine residues in the YTDIRL motif of HCPro and VPg with alanine residues (ATDIRAI) significantly reduced the interactions of both proteins with eIF(iso)4E, which compromised the PVA infection (Ala-Poikela et al., 2011; Ala-Poikela et al., 2019). In this study, the alignment of 112 potyvirus VPg sequences showed that this motif is not conserved among all potyviruses. Only 7 potyviruses carried this consensus motif within the central 89-95 amino acid region of VPg (II, Figure, 1A). However, this region was highly conserved between two potyvirus species, PVA and sweet potato feathery mottle virus (SPFMV), as previously described (Ala-Poikela, 2014), which indicated that the EIF4E-binding site is species-specific.

The VPg–eIF4E/(iso)4E interaction sites differ among potyvirus species. The amino acids involved in this interaction are unique, depending on the interaction type, and have been associated with resistance breaking (Ayme et al., 2006; Borgstrøm and Johansen, 2001; Bruun-Rasmussen et al., 2007; Gallois et al., 2010; Grzela et al., 2006; Keller et al., 1998; Léonard et al., 2000; Masuta et al., 1999; Moury et al., 2004; Nicolas et al., 1997; Rajamäki and Valkonen, 1999; Roudet-Tavert et al., 2007; Schaad et al., 1997b). Based on the nuclear magnetic resonance (NMR) structure of the VPg from PVY, which is closely related to PVA, a recent study (Coutinho de Oliveira et al., 2019) proposed a model for the PVY VPg–eIF4E complex. In this model, PVY VPg was proposed to bind with the cap-binding pocket of eIF4E, through the amino acids D111, I113, M115, Q116, L118, G119, and N121, which are found in the central domain of VPg. The study stated that this identified site in PVY VPg was the only site involved in the eIF(iso)4E interaction. However, in this study, the alignment of the PVY VPg sequence with the PVA VPg sequence, using the Jalview programme, showed that PVY VPg contains a YADIRDI sequence upstream of the interacting amino acids, which was reported by Coutinho de Oliveira et al. (2019) (Figure 3A). This finding suggested that a single amino acid substitution in the consensus eIF4E binding site in PVY VPg was able to disrupt its interaction with eIF(iso)4E. This amino substitution may have resulted in an evolutionary shift that resulted in an alternative domain becoming a functional as eIF4E binding site for PVY VPg.

Using the model described by Coutinho de Oliveira et al. (2019) as a template, an interactive model of the interaction between PVA VPg and *N. benthamiana* eIF(iso)4E was predicted in iTasser (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). The sequences were docked in the Clus pro programme (https://cluspro.bu.edu/) (Figure 3B). The model showed that interactions via the YTDIRL site in VPg occurred in close vicinity to the cap binding and cap recognition sites of eIF4E/(iso)4E (Figure 1B). Studies examining species-specific VPg–eIF4E/(iso)4E interactions have indicated that this interaction is constantly evolving in an evolutionary "arms race" between the host and the pathogen. A few positively selected point mutations in both VPg and eIF4E/(iso)4E have become functionally relevant, conferring either pathogenicity to the virus or resistance to the host. Furthermore, due to the intrinsically disordered nature of PVA VPg (Rantalainen et al., 2008), it tolerates mutations and is more prone to adaptive processes than eIF4E (Walter et al., 2019). However, in every case, a physical interaction between VPg and eIF4E/(iso)4E was found to be a crucial factor for maintaining virulence (Charron et al., 2008; Moury et al., 2014).

A



B

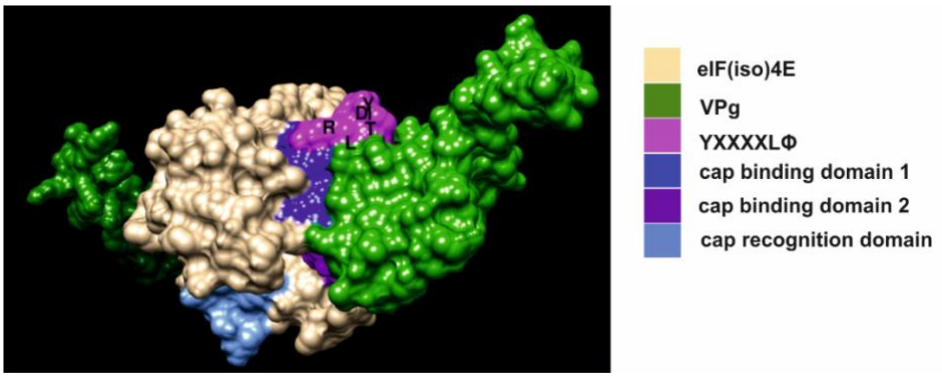


Figure 3. The YXXXXLΦ motif in PVA VPg. A) Alignment between the central domains of PVA VPg and PVY VPg, using the Jalview program. The eIF4E/(iso)4E interacting motifs/amino acids are highlighted in red. The first motif is the consensus eIF4E binding motif, which is present in PVA but presents a single amino acid mismatch in PVY (L95 to D95). The second motif shows the amino acids present in PVY and described as involved in eIF4E binding (Coutinho de Oliveira et al., 2019). Unmatched amino acids are marked with asterisks (*). B) The docking of PVA VPg and *N. benthamiana* eIF(iso)4E was performed using the Clus pro programme. Amino acids in the YXXXXLΦ motif are shown using their single-letter amino acid codes. The motifs for cap recognition and cap binding in eIF(iso)4E are highlighted using the indicated colours.

4.1.2 PVA^{VPgmut} shows reduced gene expression

Despite being one of the fundamental pre-requisites underlying infection, the VPg–eIF4E/(iso)4E interaction mechanism has not been fully elucidated. To

investigate the role played by the VPg–eIF(iso)4E interaction in viral gene expression, point mutations were made to the YTDIRL binding site of PVA VPg, as described by Ala-Poikela et al. (2019). These mutations disrupted the physical interaction between VPg and eIF(iso)4E, as determined by the yeast two-hybrid and bimolecular fluorescence assays described by Ala-Poikela et al. (2019). The mutated PVA icDNA construct was annotated PVA^{VPgmut}, and the mutated VPg protein expression construct was annotated VPg^{mut} (II, Figure 1B). Here, different phases of the virus infection cycle were examined for PVA^{VPgmut} and compared with those for wild-type PVA (PVA^{WT}) and two other PVA RNA mutants: $PVA^{\Delta GDD}$ and PVA^{CPmut}, $PVA^{\Delta GDD}$ is a replication-deficient PVA mutant, whereas PVA^{CPmut} can replicate but lacks cell-to-cell movement capacity and is restrained to the primarily transformed cells (II, Figure 1B). All of the PVA constructs used in this study contain a renilla luciferase (*Rluc*) cistron inserted between NIb and CP. Because RLUC was expressed from the 3'position of the PVA genome, it is referred to as 3' RLUC. *Agrobacterium,* carrying PVA^{VPgmut}, PVA^{WT}, $PVA^{\Delta GDD}$, and PVA^{CPmut} constructs were infiltrated into *N. benthamiana* leaves. 3' RLUC activities were estimated by using standard dual luciferase assay protocols, and the RNA expression levels were measured by quantitativereverse transcription-polymerase chain reaction (RT-qPCR) to assess viral gene expression in infected leaf samples at 3 days post-infection (dpi).

When interpreting these results, the PVA infection-based expression patterns of the vRNAs used in this study should be considered. The first round of 3' RLUC expression for all viral constructs is the result of the translation of capped vRNA transcripts from the nucleus. For a wild-type virus, further RLUC expression is likely to be translated from newly synthesised PVA^{WT} vRNA molecules, which are derived from vRNA replication and are capable of translation and cell-to-cell transmission. PVA^{CPmut} vRNA can replicate but is movement-deficient. Therefore, the gene expression associated with this construct is due to those cells that were initially infected by the *Agrobacterium* infiltration. Thus, cells infected with $PVA^{CPmut,}$ were expected to demonstrate a lower expression level than cells infected with PVA^{WT}. For the replication-deficient $PVA^{\Delta GDD}$ vRNA, both replication and movement to new cells were restricted, because non-replicating viruses are incapable of producing particles (Gallo et al., 2018) and cannot move into new cells (Dolja et al., 1994; Ivanov et al., 2003). Therefore, the expression level or RLUC associated with $PVA^{\Delta GDD}$ was expected to be the least pronounced among the examined viral mutations because the only those transcripts imported from the nucleus of transformed cells were expected to express 3' RLUC.

The result showed that the gene expression levels of PVA^{WT} were significantly higher than those for the other three constructs. $PVA^{\Delta GDD}$ showed significantly reduced gene expression levels compared with PVA^{CPmut}. The 3' RLUC activity associated with PVA^{VPgmut} was significantly reduced compared with that associated with PVA^{WT} but was increased compared with those for $PVA^{\Delta GDD}$ and PVA^{CPmut}. Similarly, the RT-qPCR results for PVA^{WT}, $PVA^{\Delta GDD}$, and PVA^{VPgmut} showed that the PVA^{WT} expression levels were much increased compared with those of the other two PVA mutants; however, the PVA^{VPgmut} RNA accumulation was significantly increased compared with that observed for $PVA^{\Delta GDD}$ RNA (II, Figure 2B and C). These results indicated that PVA^{VPgmut} might have the capacity to replicate. To further confirm the replication abilities of PVA^{VPgmut}, the minus-strand (−) RNA from leaf samples infected with PVA^{VPgmut} were detected by RT-qPCR. Leaf samples infected with PVA^{WT} were used as a positive control, and leaf samples infected with $PVA^{\Delta GDD}$ were used as a negative control in the same experiment. The results showed that the PVA^{VPgmut} samples contained an approximately five-fold higher amount of (−) RNA than the $PVA^{\Delta GDD}$ samples, although this higher amount observed for PVA^{VPgmut} was 1000-fold less than the amount observed in PVA^{WT} samples (II, Figure 2D). These data confirmed that PVA^{VPgmut} RNAs that are capped and produced from the nucleus are capable of replication.

Although PVA^{VPgmut} appears to be capable of replication, the reasons for the severely reduced gene expression observed for both 3' RLUC and RNA levels compared with PVA^{WT} remain unclear. Replicating PVA RNAs carry a VPg at their 5' ends, which serves as a protein primer for replication (Puustinen and Mäkinen, 2004). PVA^{VPgmut} RNA carries the VPg^{mut} protein at its 5'end, as demonstrated in II Figure 2A. The increased expression level observed for PVA^{VPgmut} compared with the expression level of $PVA^{\Delta GDD}$ was assumed to be due to the generation of newly derived RNA due to replication. For infection persistence, a few PVA^{VPgmut}-bearing PVA RNAs must be successfully transferred to polysomes for viral translation. However, the possibility of that some copies of PVA^{VPgmut} were able to revert to PVA^{WT} during replication could not be excluded. Therefore, whether the enhanced gene expression and minus-strand RNA accumulation were associated with the amplification of PVA^{VPgmut} RNA or a reversion to PVA^{WT} must be clarified. Western blot analysis of infected leaf samples confirmed the presence of both VPg and VPg^{mut}. A fairly small difference in the amounts of VPg expressed by PVA^{WT}, $PVA^{\Delta GDD}$, and VPg^{mut}

could be detected, which was much smaller than the observed difference observed in the corresponding RNA quantities (see Figure 2B, right panel). This difference may be because the majority of the replicating vRNA was encapsidated and not available for translation.

4.1.3 PVA^{VPgmut} produces an undetectable number of particles and fails to move systemically unless it reverts to PVA^{WT}

Potyviral particle formation is strongly associated with replication, as non-replicating viruses have been shown to be incapable of producing particles (Gallo et al., 2018). Because PVA^{VPgmut} appears to be capable of replication, particles were expected to be detectable in PVA^{VPgmut} infected samples. To determine the occurrence of particle formation by PVA^{VPgmut}, leaf samples infected with either PVA^{WT} or PVA^{VPgmut} were scrutinised under an electron microscope at 7 dpi. No particles were detected in local leaves infected with PVA^{VPgmut}, whereas the corresponding PVA^{WT}-infected leaf samples revealed the presence of particles (II Figure 3A). To confirm particle formation, immune-capture (IC)-RT-qPCR analysis was performed at 10 dpi using local and systemic leaves collected from PVA^{VPgmut}- and PVA^{WT}-infected plants. At 10 dpi, the PVA^{VPgmut}-infected local leaf samples showed no particle-encapsidated vRNA, and the values of the captured RNA were similar to those derived from mock-inoculated negative control samples. In contrast, systemically infected leaf samples that were inoculated with PVA^{VPgmut}, showed the presence of encapsidated PVA RNA at levels similar to those observed for encapsidated PVA RNA from PVA^{WT} infected leaves. The percentage of plants in which PVA^{VPgmut} caused a systemic infection was calculated by collecting one newly emerging upper leaf from each infected plant. Systemic leaf samples were collected at 10, 13, and 15 dpi. Overall, vRNA moved systemically in 26% of plants (II Figure 3B and C). Infected plants were grown in a separate chamber to avoid any potential for contamination by the wild-type virus. The PVA RNA isolated from the systemic leaves were sequenced. The results showed that all detected vRNAs in the systemically infected leaves featured the wild-type PVA sequence, which indicated that PVA^{VPgmut} had reverted to PVA^{WT} in these plants.

VPg is an intrinsically disordered protein (Rantalainen et al., 2008). The abundance of intrinsically disordered proteins is high in RNA viruses (Peng et al., 2015), as these proteins provide structural flexibility, which can enhance binding promiscuity and the capacity to accumulate mutations. RNA viruses display a high mutation rate, of approximately 10^{-3} to 10^{-5} errors/nucleotide/replication

cycle (Duffy and Holmes, 2008; Sanjuán et al., 2010), and they have a short replication cycle, which allows them to evolve rapidly under various environmental pressures (García-Arenal et al., 2001). The intrinsically disordered property observed for the central domain of VPg is maintained among potyviruses, suggesting that this region plays a functional role in virus fitness (Charon et al., 2016; Hébrard et al., 2009). Reversion mutations are common phenomena in the virus infection cycle, which allow the virus to overcome disadvantageous mutations and favour infection. In this study, 26% of plants became systemically infected, and in all of these systemically infected plants, the level of particle formation in local leaves remained under the detection limits, even after systemic infection was detected. This result indicated that a small number of PVA^{VPgmut} RNAs reverted to PVA^{WT} RNA upon replication and formed into VPg^{mut}-linked PVA^{WT} RNA. Subsequently, a few such RNAs escape degradation and become associated with the polysome and the replication complex, which then produced VPg-linked PVA^{WT} RNA. These newly reverted PVA^{WT} RNAs were capable of producing particles, and long-distance movements initiated systemic infection.

One intriguing question remains whether PVA^{VPgmut} was capable of moving systemically before the reversion. Potyviruses encode several proteins that participate in viral movement, including P3N-PIPO, CI, and CP (Carrington et al., 1998; Dolja et al., 1995; Dolja et al., 1994; Wei et al., 2010b; Wen and Hajimorad, 2010). Some functions of CP are necessary for the cell-to-cell movements of many potyviruses, such as TEV, pepper vein-banding virus (PVBV), and PVA (Anindya and Savithri, 2003; Dolja et al., 1994; Ivanov et al., 2003; Ivanov et al., 2001). CI, P3N-PIPO, CP, and vRNA localise to a canonical CI structure, which is formed by CI proteins and is anchored to PD by P3N-PIPO (Roberts et al., 1998; Rodríguez-Cerezo et al., 1997; Wei et al., 2010b). Therefore, the cell-to-cell movements of potyviral RNA can occur either via virions or viral RNP complexes, in the presence of the necessary viral proteins. VPg attached to the virion in PVA and PVY (Torrance et al., 2006) and to the VPg–eIF4E complex in LMV (Tavert-Roudet et al., 2017). However, whether the VPg–eIF4E interaction is required for particle movement remains unclear. In other studies, the association between CI and the virion tip structure was described for PVA particles (Gabrenaite-Verkhovskaya et al., 2008) and was proposed to serve as a binding site for P3N-PIPO, which is essential for virus movement (Vijayapalani et al., 2012). Although the ability of PVA^{VPgmut} RNA to bind to the PD as part of the viral RNP complex has not been previously analysed in other studies, a complex consisting of VPg–eIF(iso)4E-CI, at either the tip of

the viral particle or associated with the CP stabilising the viral RNP complex, was proposed to be necessary for loading vRNA into the PD. The lack of such complex formation in 5' VPg^{mut} capped vRNA might result in the failure of this mutant to be transported systemically, which warrants further study.

4.1.4 VPg^{mut} is unable to enhance viral RNA stability and 3' RLUC accumulation

Ectopically expressed VPg has previously been shown to enhance both PVA^{WT} and $PVA^{\Delta GDD}$ vRNA accumulation and 3' protein expression *in planta* (Eskelin et al., 2011; Hafrén et al., 2013). VPg-mediated vRNA stability is not replication-dependent; instead, VPg stabilises vRNA by loading the RNA into the polysome. However, whether the interaction between VPg and eIF(iso)4E is necessary for this function remains unknown. To determine the role played by the VPg–eIF(iso)4E interaction in the enhancement of vRNA stability and 3' protein expression, the VPg^{mut} protein construct was used to perform the following experiments. *N. benthamiana* plants were infiltrated with PVA^{WT}, $PVA^{\Delta GDD}$, and PVA^{VPgmut}, in addition to either β-glucuronidase (GUS) control, VPg, or VPg^{mut}. The sample combined with GUS represents the baseline level of viral gene expression. Infected leaf samples were subjected to the RLUC assay and qRT-PCR analysis. VPg was found to significantly enhance the expression of vRNA and 3' RLUC, for each virus tested, compared with GUS. Unlike VPg, VPg^{mut} failed to enhance vRNA accumulation and 3' RLUC expression when combined with PVA^{WT}, and the levels remained comparable to those for the GUS control. However, VPg^{mut} enhanced vRNA accumulation and 3' RLUC expression when combined with $PVA^{\Delta GDD}$ and PVA^{VPgmut}, similarly to the enhancement observed for VPg (II Figure 4). The absolute quantity of PVA^{WT} RNA and 3' RLUC accumulation were both two orders of magnitude (10^2) higher than those for the PVA^{VPgmut} and $PVA^{\Delta GDD}$ constructs. The absolute quantities of vRNA and 3' RLUC produced by PVA^{VPgmut} were unable to reach the levels observed for PVA^{WT}; instead, these levels remained similar to those observed for $PVA^{\Delta GDD}$. Capped PVA RNAs originating from the nucleus may be equally stabilised by VPg^{mut} or VPg, which indicated that VPg^{mut} could only stabilise capped vRNA, similar to VPg.

4.1.5 VPg^{mut} enhances the production of PVA-induced granules

RNA silencing is the major antiviral defence response (Ding, 2010; Pumplin and Voinnet, 2013). Recent studies have proposed that PVA induces HCPro-induced granules (PGs) that act as a safeguard to protect PVA RNA from RNA silencing (Hafrén et al., 2015). HCPro is a component of the PG and is solely responsible for initiating PG formation. Along with HCPro, other host proteins, including eIF(iso)4E, AGO1, VCS, UBP1, and P0, localise to PGs (Hafrén et al., 2015). Whether the VPg- eIF(iso)4E interaction is necessary to form PGs remains unknown. Therefore, PG formation during PVA^{VPgmu}, PVA^{WT}, and PVA$^{\Delta GDD}$ infection conditions were determined. *N. benthamiana* plants were infiltrated with PVA^{WT}, PVA$^{\Delta GDD}$, and PVA^{VPgmut}, along with either GUS, VPg, or VPg^{mut}. In each infiltration mix, yellow fluorescence protein-tagged P0 (P0YFP) was used as a marker to detect the formation of PGs under a fluorescent microscope. In the presence of VPg^{mut}, the number of PG-like P0YFP-containing foci increased significantly, for all virus constructs, compared with those in the presence of either GUS or VPg (II Figure 5). In addition, P0YFP co-expressed with PVA^{VPgmut} showed higher levels of P0YFP-containing foci than when co-expressed with PVA^{WT} or PVA$^{\Delta GDD}$. These results indicated that the presence of VPg^{mut}, either *in trans* or *in cis*, in vRNA enhances the amount of P0-containing foci.

Previous studies have shown that PGs are dynamic structures, which disappear during the later stages of infection. VPg dissolves PGs by loading the vRNA from PGs to polysomes (Hafrén et al., 2015). Interestingly, the P0YFP-containing foci that developed in the presence of VPg^{mut} appear to last longer than those that appeared in the absence of VPg^{mut}. To ensure that the P0YFP-containing foci that appeared in the presence of VPg^{mut} were not artefacts, the components of the P0YFP-containing foci were verified. The primary goal was to determine the presence of vRNA and eIF(iso)4E in PGs. PVA RNAs were identified in PGs by using a λN22- and a B-box element-based detection system. λN22 protein contains a nuclear localisation signal motif and has an affinity for the B-box RNA element of the lambda phage. λN22 is retained in the cytoplasm in the presence of B-box RNA element; otherwise, it localises to the nucleus (Schönberger et al., 2012). PVA$^{\Delta GDD}$ RNA, tagged with B-box element at the 3' UTR and known as PVA$^{\Delta GDD\ B\text{-}box}$, and a λN22-encoding sequence tagged with RFP (λN22RFP) were used to detect the presence of PVA RNA in P0YFP-containing foci, as described in Hafren et al. (2015). These two constructs were used to infect *N. benthamiana* plants, with either GUS or VPg^{mut}, and the P0YFP marker. Infected leaf samples

were observed under confocal microscopy. The results showed that a large number of the granules appeared in the presence of VPg^{mut}, but no RFP signal was visible, indicating the absence of λN22RFP and associated $PVA^{\Delta GDD\ B\text{-}box}$ in the P0YFP-containing foci. Of 76 randomly selected granules observed by eye, only 5 granules were found to contain vRNA. To calculate the co-localisation percentage between P0YFP and λN22RFP, 22 randomly selected granules were inspected by the ImageJ program. Only 20% co-localisation between P0YFP and λN22RFP was detected in the presence of VPg^{mut}, whereas 86% co-localisation was detected in the absence of VPg^{mut}. The infiltration of P0YFP and λN22RFP without the virus was used as a negative control in this experiment. No granule-like structures appeared in the negative control. In the control set, most of the RFP signal was found in the nucleus. YFP signals were primarily located in the cytoplasm and occasionally in the nucleus (II, Figure 5). Next, eIF(iso)4E tagged with RFP [eIF(iso)4ERFP] was used to determine whether the interaction between VPg and eIF(iso)4E is necessary for the association of eIF(iso)4E with the granules, as described by Hafren et al. (2015). *N. benthamiana* plants were infiltrated with either PVA^{WT} or PVA^{VPgmut}, along with eIF(iso)4ERFP and P0YFP. The infected leaf samples were then observed under confocal microscopy. The results showed that eIF(iso)4ERFP and P0YFP both localised in PGs in the presence of both PVA^{WT} and PVA^{VPgmut} constructs. However, the percentage of co-localisation was reduced in the presence of PVA^{VPgmut} when quantified by ImageJ. In a sample size n = 47, the percentage of co-localisation in PVA^{WT} expressing leaves was 86%, whereas, for PVA^{VPgmut}, the percentage had reduced to 70%. The infiltration of eIF(iso)4ERFP and P0YFP without any virus was used as a negative control. The control sample showed no granule like structures, and the eIF(iso)4ERFP and P0YFP signals were detected in both the cytoplasm and the nucleus.

Several studies have proposed the existence of a tight coupling between potyviral replication and translation (Cotton et al., 2009; Grangeon et al., 2010; Hafrén et al., 2010). However, despite the tight coupling between replication and translation, vRNAs emerging from the VRC or from the host nucleus aggregate into PGs, which appear to be involved in protecting vRNA between replication and translation. The VRCs, PGs, and translational machinery may be physically associated to ensure the secure transition from replication to translation (Hafrén et al., 2015; Lõhmus et al., 2016). The results of this study suggested that VPg–eIF(iso)4E or 5'cap–eIF(iso)4E interactions are necessary to load PVA RNA into PGs. In the presence of VPg^{mut}, a large number of P0YFP foci appeared, but only

a minute amount of vRNA co-localised with these foci. The vRNA generated from PVA^{VPgmut} replication and is associated with VPg^{mut} at their 5'ends may, therefore, lack the opportunity to become load into PGs and, instead, undergo degradation. VPg plays an important role in the maintenance of PG structure and function. VPg protects the PGs from autophagy and possibly dissolves the structure after vRNA has been successfully bound to the polysome (Hafrén and Hofius, 2017; Hafrén et al., 2015). The presence of a large number of P0YFP-containing foci that lasted for a longer period of time in the presence of VPg^{mut} may indicate that VPg^{mut} remains capable of protecting these foci from autophagy, similar to VPg, but is unable to dissolve them as efficiently as VPg^{WT}, which may be due to lack of vRNA in these structures. Whether PVA^{WT} RNA can be loaded in into PGs in the presence of VPg^{mut} and whether PVA^{VPgmut} RNA can get loaded into PGs in the presence of VPg^{WT} was not determined. The presence of VPg^{mut}, either *in trans* or at the 5'end of vRNA, does not appear to support the loading of vRNAs into PGs. The presence of eIF(iso)4E in the granules, regardless of whether cells were infected with PVA^{WT} or PVA^{VPgmut}, suggested that the large number of P0YFP-containing foci that appear in the presence of VPg^{mut} are not artefacts and likely contain the normal components of PGs, except for vRNA. How VPg^{mut} generates a large number of P0-containing foci remains to be investigated. VPg may produce a comparable number of PGs as VPg^{mut}, and these PGs may dissolve quickly as PVA RNA is loaded into polysomes. The entire process of PVA infection, including replication, PG formation, and the loading of vRNA into polysomes via VPg, is dynamic, and each step appears to be tightly coupled with the other steps. In the presence of VPg^{mut}, PGs lost their dynamic nature and were halted for a longer period of time. HCPro, which is the sole component capable of initiating granule formation, may be able to recruit all of the necessary host proteins, including eIF(iso)4E, by binding to them directly or indirectly. However, the VPg–eIF(iso)4E interaction appears to be necessary to load the vRNA into the PGs. Comprehensively, these results also validate the existence of a coordinated function between VPg, HCPro, and eIF(iso)4E to combat host-mediated silencing in PVA, as discussed in Ala-Poikela et al. (2019). A schematic representation of the infection cycle for PVA^{WT} and PVA^{VPgmut} is shown in Figure 4.

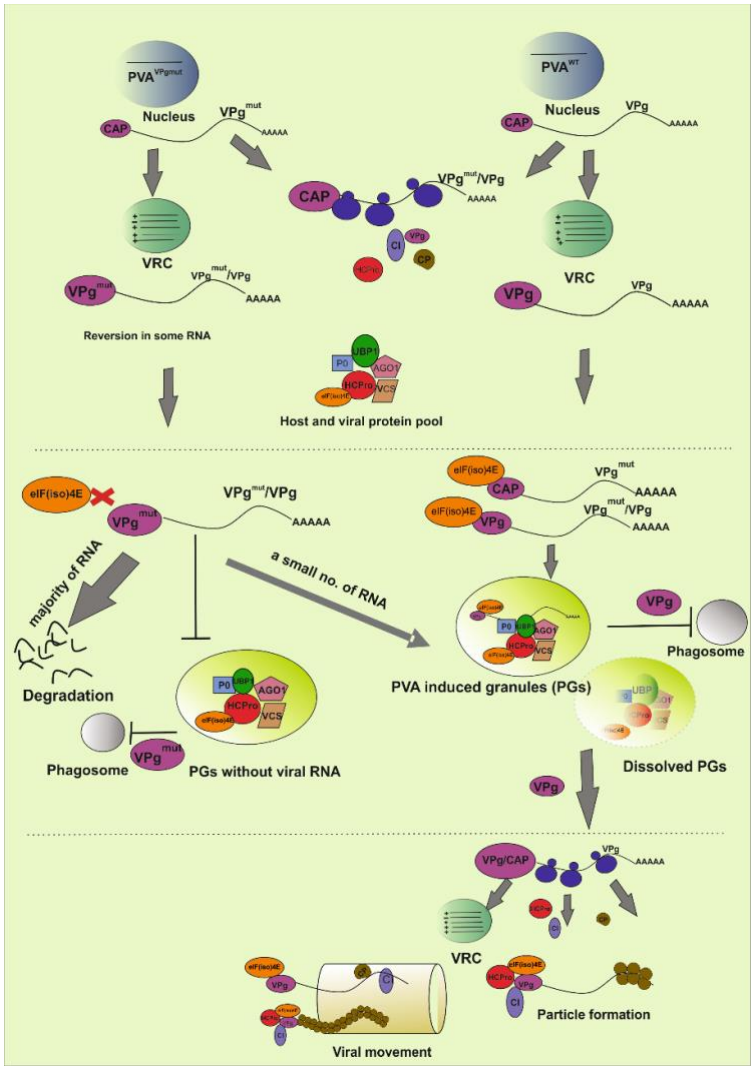


Figure 4: Schematic representation of the infection cycles of PVA^{WT} and PVA^{VPgmut}. All viral RNAs (vRNAs) produced by the nucleus contain a cap on their 5' end. vRNAs, with either VPg or a cap at the 5'end, interact with eIF(iso)4E, resulting in vRNA entering PGs, followed by translation (shown on the right side). PVA^{VPgmut}, which carrying VPg^{mut} at the 5' end of the RNA, cannot interact with eIF(iso)4E, resulting in the degradation of the majority of this vRNA (shown on the left side). A very few vRNA molecules associated with the VPg^{mut} cap become loaded into PGs. Some PVA^{VPgmut}, which undergo reversion during replication, feature a VPg^{mut} cap and PVA^{WT} RNA and may become loaded into PGs and undergo translation. These few reverted RNAs can generate wild-type VPg and carry VPg at the 5' end, similar to PVA^{WT}. The reverted PVA^{WT} virus can move systemically and establish new infections.

4.2 Article I: Dynamics of CP accumulation from the 3' side of PVA RNA is different compared with the rest of the genome

The gene expression of potyviruses is thought to occur in equimolar amounts from all parts of the genome. Therefore, translational enhancement associated with any part of the PVA ORF is considered to enhance the translation of the entire vRNA. Potyvirus produces 10 functional proteins from a single polyprotein. The discovery of the 11th protein, P3N-PIPO (Chung et al., 2008) due to transcriptional slippage (Olspert et al., 2015; Rodamilans et al., 2015), changed the concept of equimolar protein production. According to current knowledge, potyviruses produce a major product, represented by the polyprotein from P1 to CP, and a minor product, from P1 to PIPO, which results in the slightly increased production of P1 and HCPro and the slightly reduced production of P3N-PIPO compared with the production of the remaining proteins (Figure 1).

CP is a multi-functional protein that plays a significant role in viral encapsidation, movement, and gene expression, as reviewed previously (Ivanov and Mäkinen, 2012). The requirements for CP activation during the PVA infection cycle is not equal during all stages. At the beginning of the infection, during active gene expression, CP expression is maintained at low levels in the cell. CP undergoes phosphorylation by the protein kinase casein kinase 2 (CK2) (Ivanov et al., 2003), and the functional heat shock protein HSP70/HSP40 from the host chaperone pathway targets CP for proteasomal degradation (Hafrén et al., 2013). CP phosphorylation and degradation are essential for PVA replication (Lõhmus et al., 2017). The model proposed that non-phosphorylated CP blocks the translation of PVA RNA to allow the assembly of the replication complex. The transient binding of CP to vRNA allows for the assembly of the replication complex prior to its removal from vRNA by chaperone proteins. Thus, CP plays an essential role in controlling viral replication and translation during the active gene expression phase. The intriguing question remains how potyviruses switch to the production of large amounts of CP, which is required for virion formation. As CP levels increase, HSP70/HSP40 will eventually become saturated and will no longer be able to bind CP and direct CP towards degradation pathways, freeing CP to bind vRNA to cease replication and translation (Ivanov and Mäkinen, 2012; Nagy and Pogany, 2011). As the binding of CP to PVA RNA suppresses the overall translation of PVA, the inhibition of CP degradation is likely insufficient to fulfil the demands for CP. Therefore, some alternative mechanism likely exists to ensure the overproduction of CP necessary to support adequate virion

formation. VPg-mediated translational enhancement has been hypothesised to promote enhanced CP production.

4.2.1 Ectopically expressed VPg enhances viral RNA amounts equally across the genome, but protein accumulation was enhanced only for CP and 3' RLUC

In this study (I), the gene expression levels of two PVA constructs and their replication-deficient versions were examined (Figure 5). One construct contained an *Rluc* cistron, inserted on the 3' side of the PVA genome, between the NIb and CP cistrons, which was referred to as PVA^{WT}:RLUCCP in this study. Its replication-deficient counterpart was referred to as $PVA^{\Delta GDD}$:RLUCCP. The other construct contained an *Rluc* cistron on the 5' side of the PVA genome, in between the P1 and HCPro cistrons. This construct was referred to as PVA^{WT}:RLUCH, and its replication-deficient counterpart was referred to as $PVA^{\Delta GDD}$:RLUCH. The RLUC proteins expressed from these sequences were referred to as 5' RLUC and 3' RLUC, according to their position of origin in the PVA genome. To insert the *Rluc* cistron into the PVA genome with the least possible effect on infection and to ensure the complete cleavage of RLUC from the polyprotein, additional nucleotide sequences were added on both sides of the *Rluc* cistrons. The resulting 5' RLUC was surrounded by an N-terminal HHY/S cleavage site recognised, by P1 proteinase, and a C-terminal DMVYFQ/A site, recognised by NIa-Pro, to ensure the separation of 5' RLUC from P1 and HCPro, respectively. 5' RLUC is 37 kDa in size, when separated from the rest of the PVA polyprotein, as shown by western blot analysis (I, Figure 1B and C). 5' RLUC contains 5 additional amino acids at its N-terminus and 6 additional amino acids at its C-terminus. In contrast, 3' RLUC is surrounded by two DMVYFQ/A NIa-pro cleavage sites and carries 27 additional amino acids of CP sequence at its N-terminus and 18 additional amino acids of the NIb sequence at its C-terminus. The resulting 3' RLUC is approximately 41 kDa in size. The cloning strategy used for both constructs was described previously (Ivanov et al., 2003; Kelloniemi et al., 2008). Because the surrounding amino acids are not identical on the 5' and 3' sides of either RLUC, neither their activities nor their accumulation levels were compared against each other in this study.

The purpose of this experiment was to determine whether VPg-mediated translation would equally enhance all sections of the PVA genome. The PVA constructs and their replication-deficient forms were *Agrobacterium* infiltrated

into *N. benthamiana* plants, with either ectopically expressed VPg or VPg+P0. Ectopically expressed GUS was used as a control for every set. The RNA quantity and RLUC expression were measured at 3 dpi. Similar to previous findings, ectopically expressed VPg and VPg+P0 enhanced both RNA accumulation and 3' RLUC expression compared with the GUS control for both PVA^{WT}:RLUCCP and $PVA^{\Delta GDD}$:RLUCCP. The RNA accumulation of PVA^{WT}:RLUCH and $PVA^{\Delta GDD}$:RLUCH were also enhanced, similar to PVA^{WT}:RLUCCP and $PVA^{\Delta GDD}$:RLUCCP RNA. However, 5' RLUC expression was not enhanced (I, Figure 2), which indicated that vRNA expression was enhanced in the presence of VPg, regardless of the position of the *Rluc* cistron in PVA RNA. Because both PVA^{WT} and $PVA^{\Delta GDD}$ RNA were enhanced in the presence of VPg, VPg-mediated RNA enhancement is likely due to the protection of RNA from degradation, not the enhanced replication of vRNA.

We next examined whether the enhanced 3' RLUC accumulation reflects a phenomenon of biological significance. The influence of VPg on the expression of the other viral cistrons was determined. Three different proteins from both constructs were selected for this experiment, including the 5' or 3' RLUC proteins, CI, and CP. Western blot analyses showed that CI protein accumulation, expressed from either construct, did not increase in the presence of ectopically expressed VPg and VPg+P0 compared with GUS. 5' RLUC behaved similarly to CI and did not show and increased accumulation in the presence of VPg or VPg+P0. In contrast, 3' RLUC accumulation was enhanced significantly in the presence of VPg and VPg+P0 compared with GUS (I, Figure 3B and C). The amounts of RLUC observed by western blots were proportionate to the respective RLUC activities. These results suggested that VPg-mediated translational enhancement may be specific to the 3' side of the genome. This specificity could be used to enrich CP accumulation. Because 3' RLUC was located immediately upstream of CP, the increase in 3' RLUC translation may reflect the enhancement pattern for CP.

However, when CP accumulation was estimated from the same samples, no enhancement in CP accumulation was observed in the presence of VPg for either construct (I, Figure 3B and C). Because HCPro has been suggested to stabilise the CP required for particle formation (Valli et al., 2014), HCPro was ectopically expressed, in addition to VPg and VPg+P0, to determine whether HCPro had any effect on CP accumulation. A leaf sample infected with only the virus and a sample infected with the virus + HCPro were used as controls. Because the experimental conditions were altered, the expression levels of 5'and 3' RLUCs

and CI were also examined under these new conditions. The results showed that 5' RLUC and CI accumulation remained unchanged in the presence of VPg+HCPro or VPg+P0+HCPro, similar to the results obtained in the absence of HCPro. In contrast, the expression levels of 3' RLUC from PVA^{WT}:RLUCCP and CP from both constructs were enhanced significantly. Respective RLUC accumulations assessed by western blots correlated with the observed RLUC activities, and the respective CP accumulation levels visualised by western blot were proportionate with those measured by ELISA (I, Figure 4). The presence of ectopically expressed HCPro enhanced the accumulation of proteins compared with the sample infected with only the virus, as observed in I Figure 4. HCPro is a silencing suppressor, capable of enhancing PVA gene expression, to some extent. This enhancement was effective for overall PVA expression and moderate compared with the presence of VPg.

Potyviruses do not produce any sub-genomic RNA. However, we examined whether the abundance of 3' RNA increased in the cell in the presence of VPg and VPg+P0, resulting in increased 3' RLUC and CP accumulation. *N. benthamiana* plants were infiltrated with PVA^{WT}:RLUCCP and either GUS or VPg+P0. The total RNA was isolated, and cDNA synthesis was performed using two different primer sets. One set generated cDNA with the P1 specific primer from the 5' end of PVA RNA, whereas the other set generated cDNA with the CP specific primer from the 3' end of PVA RNA. The cDNA amounts were quantified by qRT-PCR. The absolute quantities of RNA in aRT-PCR were 1.5 to 4-fold higher when obtained using the P1 specific primer than when using the CP specific primer. Similar results were obtained for both GUS expressed and VPg+P0 expressed samples, which indicated that this difference might be due to the increased sensitivity of the P1 primer set compared with the CP primer set. However, when the RNA quantity of the GUS-containing sample was set to 1, the relative level of enhancement in RNA copy numbers for the VPg+P0-containing sample was approximately 17-fold higher, regardless of which primer set was used (I, Figure 4A and B), which indicated that the VPg+P0 sample does not contain excess 3' PVA RNA fragments. The level of RLUC expression in the same samples was approximately 10-fold higher in the VPg+P0-containing sample compared with the GUS-containing sample. These data demonstrated that in the presence of VPg+P0, the total PVA RNA level was enhanced. The fold difference in RNA expression levels was 17-fold that of the control, whereas the fold difference of 3' RLUC expression was only 10-fold that of the control, which

is likely because total RNA levels include both translatable and non-translatable RNA, whereas RLUC expression is only indicative of the translatable RNA level.

The accumulation of a given protein depends on the sum of its production and degradation rates. RLUC and firefly luciferase (FLUC) are neutral proteins to PVA and plants. RLUC activity and concentration are maintained at a 1:1 ratio (https://fi.promega.com/resources/product-guides-and-selectors/protocols-and-applications-guide/bioluminescent-reporters/). Both RLUC and FLUC feature short half-lives of 4.5 hrs, and 3.5 hrs, respectively. This feature allows these proteins to report dynamic gene expression responses (Thorne et al., 2010). The result showed that VPg enhanced the activity and accumulation of 3' RLUC but not 5' RLUC. Based on the expected half-life of RLUC, RLUC activity tends to represent current viral gene expression rather than viral protein accumulation over the long term. Because neither 3' RLUC nor 5' RLUC is expected to affect host gene expression or PVA infection, no biologically relevant modifications are expected to influence their expression. However, some biochemical effects cannot be ruled out. Two particular biochemical effects must be considered to avoid misrepresentation when examining 5' RLUC and 3' RLUC activities. One is the possibility that differences in 5' RLUC and 3' RLUC processing occur and the other is their relative stabilities. Western blot analysis revealed that the extra amino acids associated with 3' RLUC did not affect 3' RLUC processing from the polyprotein. In contrast, 5' RLUC processing appeared to be partially affected, leaving a small amount of unprocessed RLUC, which appeared as a higher molecular weight band at the top of the SDS gel (I, Figure 1C). Due to modifications, 5' RLUC and 3' RLUC may be associated with different levels of stability, which could theoretically affect the results; however, this outcome would require the destabilisation of 5' RLUC and the stabilisation of 3' RLUC only in the presence of ectopically expressed VPg, which is unlikely. In the presence of VPg, both the accumulation and activity of 3' RLUC increased, whereas 5' RLUC showed no changes in either accumulation or activity compared with the GUS control, despite the RNA level increasing substantially.

Higher CP production relative to the other potyviral proteins has not been reported thus far for potyvirus. The present study has approached this question. Whether the overexpression of VPg enhances CP production or promotes CP accumulation by blocking the proteasomal degradation of CP remains unclear. Because 3' RLUC concentration increased along with CP, the accumulation of CP may be due to enhanced translation, rather than CP stabilisation by VPg.

However, clear evidence of enhanced translation from the 3' side of the PVA genome in the presence of VPg has not yet been reported. In our study, 5' RLUC and 3' RLUC, as reporters for the level of protein production from the 5' and 3' side of the genome, respectively, showed different patterns of gene expression associated with the two sides of the potyvirus genome.

The finding that viral proteins encoded by the PVA genome may be produced at different ratios would represent a sharp contradiction from our current understanding of potyvirus protein production and remains a matter of debate. CI is another PVA protein, produced from the centre of the PVA genome. In this study, it is used as a representative of the remaining PVA cistrons, other than CP. CI, produced from the centre of the PVA genome, forms pinwheel-shaped inclusion bodies, which act as markers of potyvirus infection (Edwardson, 1974). The pinwheel structures are formed during the early infection period when viral gene expression remains in the active stage (Roberts et al., 1998). CI, together with P3N-PIPO, forms a conical structure in the PD that is essential for viral movement (Wei et al., 2010b). CI assembly may stabilise and increase the half-life of CI. Therefore, the amount of CI detected by western blot likely reflects the amount of CI accumulated over time, rather than reflecting the rate of production. The CI concentration remained constant in the presence of VPg, which suggests that the effect of VPg is particular to CP expression and the positions of the *Rluc* cistrons within the construct but does not influence the other viral cistrons.

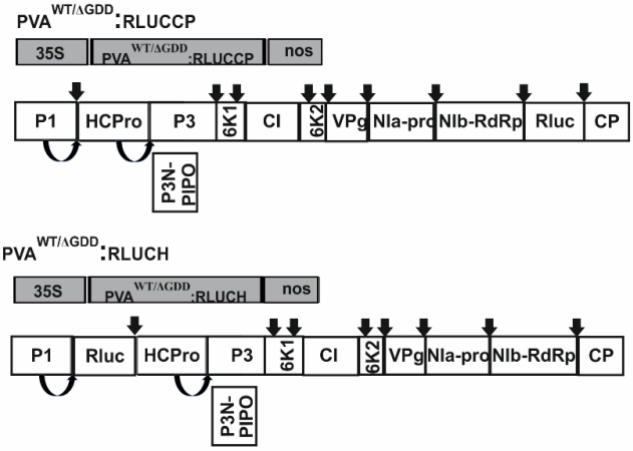


Figure 5: Schematic representation of the PVA^{WT} and PVA$^{\Delta GDD}$ constructs with *Rluc* cistrons inserted either on the 3' or 5' sides of the genome. PVA$^{WT/\Delta GDD}$:RLUCCP expresses 3′ RLUC between the NIb and CP cistrons of PVA RNA. PVA$^{WT/\Delta GDD}$:RLUCH expresses 5′ RLUC between the P1 and HCPro cistrons of PVA RNA.

4.2.2 Comparing the accumulation of individual viral proteins produced by PVA^{WT} and $PVA^{\Delta GDD}$ revealed greater differences in the accumulated levels of 3'-RLUC and CP than in those for 5'-RLUC and CI.

To understand whether the VPg-mediated enhancement of CP accumulation occurs during the normal course of infection, the pattern of protein accumulation was examined in naturally infected samples. For this purpose, *N. benthamiana* plants were infiltrated with only viral constructs: PVA^{WT}:RLUCCP, $PVA^{\Delta GDD}$:RLUCCP, PVA^{WT}:RLUCH, or $PVA^{\Delta GDD}$:RLUCH. The protein expression levels of 5' and 3' RLUCs, CI, and CP from all constructs were examined by western blot at 5 dpi. The protein amounts expressed by the respective $PVA^{\Delta GDD}$ constructs were considered the baseline levels of minimally expressed proteins. As expected, 5' RLUC, CI, 3' RLUC, and CP expression levels were increased in samples infected with PVA^{WT} than those in samples infected with $PVA^{\Delta GDD}$. Comparison of individual protein expression levels between the replicating and non-replicating viruses showed that the 5' RLUC and CI expression level ratios between PVA^{WT} and $PVA^{\Delta GDD}$ samples were much lower than the corresponding ratios for 3' RLUC and CP expression levels (I, Figure 6), which indicated that PVA protein accumulation/production was not equal across all parts of the genome. CP and 3' RLUC were expressed at higher levels by PVA^{WT} over time than 5' RLUC and the central CI protein. As CP accumulation remained low at the onset of PVA infection, to ensure viral gene expression (Besong-Ndika et al., 2015; Hafrén et al., 2010; Lõhmus et al., 2017), the emergence of increased CP accumulation in PVA infection could be observed. In a similar experiment, the progress of 5' and 3' RLUC and CP accumulation in samples infected with PVA^{WT} and $PVA^{\Delta GDD}$ were compared at different time points, ranging from the early to late infection stages. The expression levels of 5' and 3' RLUC were compared at 3, 4, 5, and 6 dpi and CP accumulation was examined at 3, 5, and 6 dpi. The results showed that both the ratios for 3' RLUC and 5' RLUC were moderate at 3 and 4 dpi, after which the 3' RLUC ratio increased significantly moving towards the later stage of infection. At 6 dpi, the ratio of 3' RLUC accumulation in wild-type vs ΔGDD viruses was approximately 560-fold (I Figure 7), but the corresponding difference in the 5' RLUC ratio was only 27-fold. This result suggested that the amount of 3' RLUC accumulated more pronouncedly than the amount of 5' RLUC during the later stages of infection. When the CP ratios were compared between PVA^{WT} and $PVA^{\Delta GDD}$ constructs for both PVA:RLUCCP and PVA:RLUCH viruses, the CP ratios for

both constructs were comparable at every time point, which suggested that CP accumulation occurred with similar dynamics for both viruses, irrespective of the *Rluc* position inside the genome.

The first round of protein expression for both PVA^{WT} and $PVA^{\Delta GDD}$ occurs due to the appearance of the capped mRNA from the nucleus. The level of gene expression that occurs in cells expressing the replication-deficient virus is limited to the protein expressed by the capped mRNA, which is incapable of moving or infecting new cells. Therefore, $PVA^{\Delta GDD}$ expression increased from 2 dpi to 5 dpi before reaching saturation (I, Figure 8). For the PVA^{WT} virus, the first round of gene expression occurs through the same mechanism as that for $PVA^{\Delta GDD}$; however, the increase in gene expression observed later is due to replication.
Because the RNA levels appeared to remain comparable between PVA^{WT}:RLUCCP and PVAWT:RLUCH and between $PVA^{\Delta GDD}$:RLUCCP and $PVA^{\Delta GDD}$:RLUCH, the increase in protein accumulation associated with the 3' end may be due to changes in translation. The enhanced expression levels of 3' RLUC and CP were detected in the presence of VPg alone or in the presence of VPg combined with auxiliary proteins at 3dpil however, similarly enhanced levels of 3' RLUC and CP expression were observed even without the additional expression of VPg, under natural infection conditions, at 5 and 6 dpi. These two phenomena are likely to be due to the same mechanism. Towards the later stages of infection, due to several rounds of gene expression, all viral proteins, including VPg and HCPro, are likely to accumulate sufficiently to facilitate enhanced 3'end CP production. The presence of ectopically expressed VPg and HCPro mimics the situation observed during late infection. However, $PVA^{\Delta GDD}$:RLUCCP, which showed the enhancement of 3' protein accumulation in the presence of VPg, did not display increased 3' protein accumulation under natural conditions as a function of time. This virus may not be able to express a sufficient amount of either VPg or HCPro under natural conditions due to the limited production of proteins from the replication-deficient viral construct.

4.2.3 Viral RNA, 3' RLUC, and CP accumulate with different dynamics than 5' RLUC and CI during the course of PVA infection

Next, we examined the dynamic patterns of vRNA and protein accumulation from the early to late infection stages. *N. benthamiana* plants were *Agrobacterium* infiltrated with PVA^{WT}:RLUCCP or PVA^{WT}:RLUCH constructs. The vRNA, CI,

RLUC, and CP amounts were quantified daily between 3–7 dpi. The results showed that the RNA amounts increased at similar rates for both constructs. An approximately 10-fold difference in RNA amounts was found between 3 and 7 dpi, for both constructs. The RNA amount reached its maximal value at 6 dpi and plateaued afterwards (I, Figure 8A and B). 5' RLUC activity and accumulation was higher at the beginning of the experiment and continued to increase until 5 dpi. 3' RLUC, began from a much lower activity level and a smaller accumulation level but grew continuously until 7 dpi. The maximum fold difference between 3 and 7 dpi for 5' RLUC was 2.8, whereas for 3' RLUC the maximum fold difference was 112 for the same time period. The more significant fold difference observed for 3' RLUC was due to the very small level of 3' RLUC accumulation at the beginning of the infection. The absolute quantity of 5' RLUC was higher than that for 3' RLUC at 3 dpi. These data suggested that 5' and 3' RLUC accumulated with different dynamics (I, Figure 8 C and D). When the CP amounts were compared using both ELISA and western blot analysis, the CP levels produced by both constructs increased steadily from 3 to 7 dpi. The CP accumulation observed for both constructs was comparable, with that from PVA^{WT}:RLUCH being only 1.2-fold higher than that from PVA^{WT}:RLUCCP, which suggested similar levels of CP accumulation from both constructs (I, Figure 8 E and F). Western blot analysis of CI suggested that CI accumulation from both constructs increased at similarly rates until 5 dpi and remained constant after that. Taken together, these data indicated that the PVA RNA amount increased through 6 dpi and the levels of 3' RLUC and CP increased through 7 dpi, whereas the 5' RLUC and CI amounts remained constant after 5 dpi (I, Figure 8G and H). These data suggested that 5' RLUC and CI accumulate at different rates than 3' RLUC, CP, and PVA RNA.

A clear difference was observed between the 5' and 3' gene expression dynamics from a single PVA construct. These dynamics were further studied by generating a new construct containing two reporter cistrons, one on the 5' side and the other on the 3' side of the PVA genome. The gene construct was generated by inserting the same 5' *Rluc* cistron (5' RLUC), in between the P1 and HCPro cistrons, similarly to the PVA:RLUCH construct. Another reporter gene, firefly luciferase (*Fluc),* was inserted on the 3' side (3' FLUC), between the NIb and CP cistrons, similar to the position used for 3' *Rluc* in PVA:RLUCCP. This PVA construct was named PVA^{WT}:RF and produces two reporter proteins, 5' RLUC and 3' FLUC (I, Figure 9A). The construct was *Agrobacterium* infiltrated at 0.01 OD_{600}, which allows for the virus to spread cell-to-cell. The luciferase activities were

plotted as a function of time from 2 to 5 dpi. In line with our previous results, 5' RLUC accumulation was pronounced starting at 2 dpi and increased at a moderate rate through 5 dpi. In contrast, 3' FLUC accumulation was minute at 2 dpi but increased at a much faster rate than did 5' RLUC until 5 dpi. The fold difference between 2 dpi and 5 dpi for 5' RLUC was 42-fold compared with 960-fold for 3' FLUC. Thus, the fold difference in 3' FLUC enhancement was 23-fold the fold difference in 5' RLUC activity from 2 to 5 dpi (I, Figure 9B). Collectively, this result suggested that PVA proteins accumulate non-synchronously during the infection process. 5' RLUC and CI accumulated at a faster rate at the beginning of the infection, whereas 3' FLUC and CP accumulated less rapidly at the beginning of the infection, but the accumulation became enhanced during the later stages of infection.

In many plants and animal DNA viruses, including adenoviruses (Nevins, 1991), herpes viruses (Menegazzi et al., 1999), SV40s (Khoury and May, 1977), and begomoviruses (Shimada-Beltrán and Rivera-Bustamante, 2007), a clear separation between structural and non-structural protein production has been observed during different phases of the infection cycle. In these viruses, early genes encode proteins required for replication and viral gene expression, whereas late genes encode structural proteins. The protein expression pattern associated with Brome mosaic virus (BMV) infection represents a good example among plant RNA viruses for how the tight regulation of CP gene expression defines the spatial-temporal separation of early and late gene expression. During the early stages of BMV infection, when CP amounts are limited, CP enhances replication. As CP levels increase towards the later stages of infection, CP inhibits replication (Chapman and Kao, 1999) and viral translation in a concentration-dependent manner (Yi et al., 2009a; Yi et al., 2009b), similarly to CP function in PVA (Besong-Ndika et al., 2015).

Viruses utilise various strategies for the translation and regulation of viral protein production, as reviewed previously (Miras et al., 2017). The initiation of viral translation incorporates various strategies, such as scanning, shunting, and internal ribosome entry sites (IRESs), which were reviewed previously (Jan et al., 2016). This regulation is crucial to maintaining the correct amounts of each viral protein necessary for the various phases of infection. Human immunodeficiency virus (HIV) uses polyprotein production as the primary translational strategy, but HIV uses various methods to produce the structural protein Gag (Deforges et al., 2017). For some carmoviruses, in the *Tombusviridae* family, CP has also been

found to be produced from an IRES located immediately upstream of CP, in addition to a sub-genomic RNA ORF, which is more typical for CP production (Fernández-Miragall and Hernández, 2011; Koh et al., 2003).

In the PVA genome, reporter cistrons associated with CP were also enhanced in higher amounts. Both 3' RLUC from the PVAWT:RLUCCP construct and 3' FLUC from the PVAWT:RF construct were cloned similarly, maintaining a stretch of 81 nucleotides between the N-terminal CP cistron and the *3' Rluc*/*Fluc* cistron. The increased CP production observed may be associated with the first 81 nucleotides of CP. The presence of alternative AUGs and a suitable Kozak sequence (Kozak, 1989) for CP production would support this idea. A long-distance RNA-RNA interaction also serves as a regulatory function for IRES-mediated translation (Jang et al., 1988; Pelletier and Sonenberg, 1988). Often, viral gene expression from positive-strand viruses occurs due to vRNA circularisation, which involves direct RNA–RNA contacts between the 5′ and 3′ ends and RNA-binding protein bridges, as reviewed previously (Martínez-Salas et al., 2015). In many plant RNA viruses, direct RNA–RNA interactions between the 3′ and 5′ UTR sequences of the genome can regulate the initiation of cap-independent translation (Miller et al., 2007; Rakotondrafara et al., 2006; Shen and Miller, 2007). In the case of potyviruses, eIF4G binds to the pseudoknots of the 5' UTR and interacts directly with the PABP from the 3' poly(A) tail (Gallie, 2001). This interaction can promote the circularisation of PVA RNA, bringing the CP cistron closer to the translational initiation complex and recruiting additional CP translation. In the light of these examples, an unconventional mechanism for CP translation may exist for potyvirus. Some hypothetical models are presented in Figure 7 to demonstrate how enhanced CP translation and particle formation could occur. Alternatively, a mechanism that inhibits CP production at the beginning of the infection that then inhibits the production of replication proteins during the later phase of in infection may also exist. The testing and validation of both possibilities require further investigation.

4.3 Article III: Functions of HCPro and CP in RNA silencing suppression, viral protein accumulation, and virion formation in potato virus A infection

HCPro is involved in multiple functions, including silencing suppression, virion formation, and viral translation. The interconnection and implementation of these

functions through the interplay of HCPro as a key regulator of RNA silencing suppression, viral protein accumulation, and virion formation were examined.

4.3.1 PVA HCPro cannot stabilise CP alone

Valli et al., 2014 showed that in PPV, HCPro provided stability to CP when infected leaf samples were subjected to an *in vitro* degradation assay. Whether PVA HCPro could similarly provide stability to CP *in vivo*, in the absence of other PVA proteins or vRNA, was examined. To determine CP stability, *N. benthamiana* plants were infiltrated with expression constructs for wild-type HCPro or the mutants $HCPro^{WD}$, $HCPro^{4Ebd}$, and $HCPro^{SD}$, along with a CP expression construct, at OD_{600} = 0.3. $HCPro^{WD}$ features a mutation in the WD40 protein-binding site (De et al., 2020). This mutation the ability of HCPro to associate with VCS. VCS is a WD40 domain-containing scaffolding protein that is found in protein complexes that are active in RNA metabolism (Xu et al., 2006). VCS is a component of PGs and participates in PVA translation, in combination with VPg (Hafrén et al., 2015). $HCPro^{4Ebd}$ is deficient in eIF4E binding (Ala-Poikela et al., 2011), and $HCPro^{SD}$ is deficient in the suppression of RNA silencing (Hafrén et al., 2015). GUS was overexpressed as a negative control in this experiment. The infected leaf samples were collected at 3 dpi and were subjected to a degradation assay, similarly to that described previously (Gallo et al., 2018; Valli et al., 2014). After one hour of incubation, CP levels were detected using western blot analysis. The results showed that CP was degraded within one hour of incubation at room temperature in the presence of all tested HCPro constructs, suggesting that neither HCPro nor any of the HCPro mutants were able to stabilise CP (III, Figure 1). This result indicated that HCPro was unable to stabilise CP in a virus-free host system.

4.3.2 Several viral RNA silencing suppressors can complement PVA gene expression but not CP stabilisation in the absence of HCPro

Whether the CP stability provided by HCPro could be complemented by providing HCPro or other silencing suppressors *in trans* was then examined. Some functions, such as potyvirus amplification and movement, can be complemented by supplying HCPro *in trans* (Hafrén et al., 2015; Kasschau et al., 1997). Previously, $HCPro^{WT}$ was shown to enhance the expression of 3' RLUC in a $PVA^{\Delta HCPro}$ virus (Hafrén et al., 2015). The silencing suppressors of other viral

groups, including cucumoviral 2b, tombusviral P19, and potexviral P25, were also shown to complement the gene expression of $PVA^{\Delta HCPro}$ (Hafrén et al., 2015). To determine whether CP stability could be enhanced by other silencing suppressors, *N. benthamiana* plants were infiltrated with a viral construct lacking the HCPro cistron ($PVA^{\Delta HCPro}$), along with HCPro, $HCPro^{WD}$, or a heterologous viral silencing suppressor (VRS) 2b. GUS was co-infiltrated as a negative control, and PVA^{WT}, alone, was infiltrated as a positive control. The results showed that 2b was able to complement the expression of $PVA^{\Delta HCPro}$, similar to HCPro, (III Figure 2A), similar to the results reported by Hafren et al. (2015). $HCPro^{WD}$ was found to be a less efficient silencing suppressor than HCPro (De et al., 2020), and it also complemented PVA gene expression less efficiently than HCPro. VRNA was quantified by qRT-PCR. Except for the 2b complementation condition, the relative increases in 3' RLUC activity and vRNA levels were correlated. However, in the case of 2b complementation, the increased 3' RLUC activity was derived from a lower RNA amount (III, Figure 2), which may be due to the encapsidation of vRNA in virions in the presence of HCPro. To quantify virion formation under the same conditions, the samples were subjected to immune-capture qRT-PCR (IC-qRT-PCR). The data showed that particle formation could only be achieved in the presence of HCPro, and 2b was unable to support particle formation for the $PVA^{\Delta HCPro}$ virus, despite being able to complement gene expression. To assess CP stability, the same leaf samples were subjected to a degradation assay. Leaf extracts were incubated for one hour at room temperature, and CP levels were assessed by western blot at the zero time point and after one hour. The results showed that in lysates that contain either PVA^{WT} or $PVA^{\Delta HCPro}$ complemented with HCPro, the CP levels remained stable before and after incubation. The samples that contained $HCPro^{WD}$ had a faint but detectable amount of CP at the zero time point, but CP was not detectable after one hour of incubation. In the presence of GUS or 2b, CP accumulation remained below the detection limit, even though viral gene expression levels were complemented by 2b. These results revealed that 2b was incapable of stabilising either free CP or PVA particle-bound CP. Thus, these data suggested that viral gene expression was dependent on silencing suppressor capacity, in general. Therefore, viral gene expression can be complemented either fully or partially by heterologous silencing suppressors. In contrast, PVA CP stabilisation is a separate function that can only be achieved by the presence of HCPro. These results were in line with the results presented for PPV by Valli et al. (2014), who showed that PPV particle formation requires a homologous silencing suppressor (Valli et al., 2014). Several motifs within HCPro are involved in various functions (Plisson et

al., 2003). The motif required for virion formation in PPV (Valli et al., 2014) is located in the central domain of HCPro, between amino acids 100–300. This motif consists of two arginine residues, R234 and R235, and a histidine residue, H236, within PPV HCPro. The central region of PVA HCPro contains two similarly located arginine residues, R239 and R240, which may contribute to PVA particle formation. However, the functions of these residues for PVA particle formation were not verified in this study. Further investigation remains necessary to identify the region of HCPro that contributes to PVA particle formation. Mutations made to a similar region in TEV, another potyvirus, affected systemic movement (Kasschau et al., 1997), possibly associated with disrupted particle formation, which hampers systemic movement; however, this possibility requires further investigation. HCPro^{WD} is a weak silencing suppressor that could partially rescue HCPro functions but was unable to stabilise CP or form particles. The observed degradation of CP in the presence of HCPro^{WD} and the failure to effectively accomplish particle formation suggests that the interaction between HCPro and VCS is necessary for both CP stability and particle formation.

4.3.3 HCPro relieves the CP-mediated block in viral gene expression

The level of PVA CP is tightly regulated during the different stages of infection. During the early infection stage, the CP amount is maintained at a low level because CP inhibits PVA gene expression at the translational level (Besong-Ndika et al., 2015; Hafrén et al., 2010). In contrast, a high level of CP is required for virion formation. PVA virion formation has been proposed to be initiated by CP-CP interactions on the viral genome during translation (Besong-Ndika et al., 2015). Whether the CP-mediated translational block occurs at all parts of the genome and whether HCPro influences the CP-mediated translational block to favour virion formation were investigated. Previously, the overexpression of CP was shown to inhibit PVA gene expression, which was examined by estimating 3' RLUC expression (Besong-Ndika et al., 2015). In Article I, VPg overexpression was shown to specifically enhance the accumulation of RLUC and CP produced from the 3' end of vRNA. This effect was determined by estimating the expression levels of RLUC from two different PVA constructs. One construct inserted an *Rluc* cistron before HCPro (PVA^{WT}:RLUCH), whereas the other construct inserted the cistron between the NIb and CP cistrons (PVA^{WT}:RLUCCP; see Figure 5). The PVA^{WT}:RLUCCP construct is referred to as PVA^{WT} in the present study.

N. benthamiana plants were infiltrated with *Agrobacterium* carrying either PVA^{WT}:RLUCH or PVA^{WT}:RLUCCP and *Agrobacterium* carrying either the GUS control or a PVA CP expression construct. 5' and 3' RLUC expression was determined at 3 dpi. The results showed that CP inhibited RLUC expression at comparable levels for both constructs (III, Figure 3). This finding suggested that CP blocks translation from the entire PVA RNA (Besong-Ndika et al., 2015).
Because CP blocks PVA gene expression across all parts of the genome, the influence of HCPro on the CP-mediated translational block was determined using only the PVA^{WT}:RLUCCP construct, referred to as PVA^{WT}. Two other PVA mutant constructs, PVA^{CPmut} and $PVA^{\Delta GDD}$, which were described in Section 5.1, were also used in this experiment. PVA^{CPmut} and $PVA^{\Delta GDD}$ RNAs carry an *Rluc* cistron between the NIb and CP cistrons, similar to PVA^{WT} RNA. *N. benthamiana* plants were infiltrated with these viral constructs and the PVA CP and HCPro expression constructs, either together or separately. The GUS overexpression construct was used as a control in every case. Gene expression was measured at 3 and 6 dpi. The results showed that the overexpression of CP strongly inhibited PVA^{WT} gene expression for all the constructs (III, Figure 3). The overexpression of HCPro showed no effects on PVA gene expression. The estimated RLUC value in the presence of overexpressed HCPro was comparable to the RLUC value of the GUS control for $PVA^{WT,}$ but these values were slightly increased for PVA^{CPmut} and $PVA^{\Delta GDD}$ (III, Figure 4). The co-expression of CP with HCPro rescued viral gene expression from PVA^{WT}, PVA^{CPmut}, and $PVA^{\Delta GDD}$ at 3 dpi which were blocked by CP either partially or completely in parallel experiments (III, Figure 4C, D). CP expression levels were verified by western blot in the PVA^{WT} infected sample. At 6 and 9 dpi, CP accumulation was detectable in all the cases, except for the sample overexpressing CP. Particle formation from PVA^{WT}-infected samples at 9 dpi was assessed by IC-qRT-PCR. The amount of RNA captured in all samples was proportionate to the level of gene expression observed at 6 and 9 dpi. During the later stages of infection, the binding of CP to vRNA blocks viral gene expression and initiates encapsidation. In the absence of HCPro, vRNA cannot be encapsidated and instead undergoes degradation. Stabilisation by HCPro is necessary for successful particle formation.

4.3.4 Replication is required for particle formation

Next, whether non-replicating viruses can produce particles in the presence of sufficient CP and HCPro levels in infected cells were examined. This experiment

utilised the non-replicating virus PVA$^{\Delta GDD}$ and two other PVA mutants, PVA^{AAA} and PVA^{ADA}, which are either non-replicating or display severely compromised replication abilities due to mutations in the CK2 phosphorylation site of PVA CP (Lõhmus et al., 2017). *N. benthamiana* plants were infiltrated with these viruses, and RLUC activity was measured at 3 dpi. The RLUC activities were comparable among these samples. The CP expression levels of these samples were examined by western blot. CP was detectable in each case (III, Figure 5A and B). The degradation assay was performed using the same leaf samples at 0, 30, and 60 min. The results showed that CP was degraded completely after 60 min of incubation for all examined viruses. By 30 min, the CP expressed by PVA$^{\Delta GDD}$ and PVA^{AAA} was degraded completely, whereas a faint CP band remained detectable for PVA^{ADA} after 30 min of incubation, which indicated partial stability or some particle formation by PVA^{ADA}. In the next set of experiments, only PVA$^{\Delta GDD}$, combined with GUS control, CP, or CP + HCPro, were used. Viral gene expression, RNA accumulation, and virion formation were estimated at 5 dpi. The expression levels observed for PVA^{WT}, which acted as a positive control, and that observed for PVA^{WD}, which can replicate but cannot produce particles, were compared. The results showed that 3' RLUC expression from PVA$^{\Delta GDD}$ was severely reduced in the presence of CP. The co-expression of HCPro combined with CP restored 3' RLUC expression, from as low as 10% to total restoration. Because the CP-mediated block is concentration-dependent (Besong-Ndika et al., 2015), the restoration of the block by HCPro depended on the respective levels of expressed CP and HCPro. RNA levels were quantitated at 5 dpi, which revealed that no vRNA was detected in the presence of overexpressed CP or in the presence of CP + HCPro (Figure 5). IC-qRT-PCR from the sample showed a similar effect, with no particle formation observed for PVA$^{\Delta GDD}$, even in the presence of excess CP and HCPro. For comparison, PVA^{WD}, which can replicate, produced nearly 10-fold more 3' RLUC and RNA than GUS but failed to produce any stable virions. These results suggested that some other factors are likely to be involved in stable virion formation, in addition to replication. The PVA^{WD} genome harbours mutations in the sequence encoding the WD40 protein-binding site of HCPro, which prevents the virus from interacting with VCS. The interaction between HCPro and VCS is necessary for RNA silencing suppression, translation, encapsidation, and the systemic spread of PVA infections (De et al., 2020).

A number of studies have revealed that a group of RNA binding proteins (RBPs) from both the host and virus necessary to provide stability to the vRNA bound to

CP (De et al., 2020; Hafrén et al., 2015; Ivanov et al., 2016; Pollari et al., 2020) HCPro, the primary initiator of PG formation, in addition to PVA RNA and various host and viral proteins, including VCS, AGO1, eIF(iso)4E, P0, and UBP1, form PGs in PVA-infected cells (Hafrén et al., 2015). In another line of research (Ivanov et al., 2016), the initiation of viral translation was found to require a ribosome-associated 5' RNP complex, consisting of at least HCPro, VPg, CI, eIF(iso)4E, eIF(iso)4A, eIF(iso)4G, and the silencing component AGO1. During the final stage of PVA RNA maturation, i.e. during particle formation, several proteins, including CI (Gabrenaite-Verkhovskaya et al., 2008), VPg (Torrance et al., 2006), and the host protein eIF(iso)4E (Tavert-Roudet et al., 2017), have found to be associated with virions.

Several lines of evidence have indicated that virus replication is associated with viral translation, and many translation-associated proteins are localised to the VRC (Beauchemin et al., 2007; Beauchemin and Laliberté, 2007; Cotton et al., 2009; Hafrén et al., 2013; Lõhmus et al., 2016; Thivierge et al., 2008). PGs serve as the intermediate structures between PVA replication and translation and are closely associated with VRCs (Hafrén et al., 2015). Many but not all viral and host proteins are common between the VRC, PG, ribosome-associated 5′ RNP complex, and virions. HCPro acts as a master component during PG formation, safeguarding vRNA from replication to translation (Hafren et al., 2015). VPg, in turn, acts as a master component that maintains replication and enhances translation (Eskelin et al., 2011). The molecular reallocation of RBPs can enable vRNA to move from one stage to the next. Recent studies have shown that silencing AGO1 and VCS can reduce PVA infection (Hafrén et al., 2015). Interestingly, specific mutations on HCPro proteins that disrupt interactions with AGO1 and VCS also prevent particle formation(De et al., 2020; Pollari et al., 2020). In article II, the lack of interaction between VPg and eIF(iso)4E resulted in the failure to produce particles by the replicating PVA^{VPgmut} virus. VPg interacts with itself and has multiple partners (Jiang and Laliberté, 2011). The stage of the PVA infection cycle at which these proteins get associated with virions is not clear. The molecular reallocation of RBPs likely begins at the onset of viral replication and continues until virion formation has been achieved, which protects vRNA from degradation and assists the progression of PVA RNA through each phase of the potyvirus infection cycle (Figure 6). VPg is present on the 5' end of vRNA and may act as the centre of viral interactions, through which multiple interacting partners from both the host and the virus are established. The presence of a cap on the 5'end of $PVA^{\Delta GDD}$ RNA instead, of the VPg that is typically found

at the 5'end of PVA^{WT} RNA, may prevent the formation of an interaction network that depends on VPg, why may explain why non-replicative viruses fail to produce viral particles. Towards the later stages of infection, when all viral proteins are abundant, VPg may specifically enhance CP production, for both replicating and non-replicating viruses. A complex consisting of host and viral proteins, established at the 5' UTR, may be required for vRNA to produce stable particles. A schematic presentation showing increased CP production, 5' UTR complex formation, and the initiation of virion formation is presented in Figure 7.

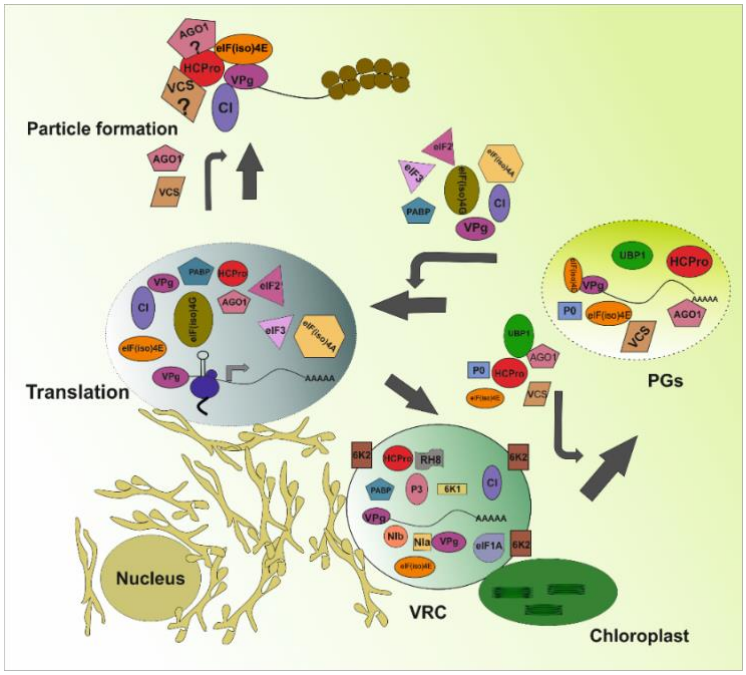


Figure 6: Re-allocation of viral RNA (vRNA)-associated proteins at different phases of the potyvirus infection cycle. Interactions among proteins are not shown here. The 6K2 membrane-spanning protein initiates virus replication complex (VRC) formation at the ER membrane by recruiting vRNA and other proteins. VRC fuses with chloroplast membrane, where active replication begins. vRNA moves from the VRC to the PVA-induced granules (PG). HCPro is a key component of the PG, which gathers other host and viral proteins into PGs. The VPg–eIF4E/(iso)4E interaction necessary to load vRNA into PGs. vRNA moves from PGs and becomes loaded into polysomes for translation, with the aid of VPg. A set of host and viral proteins form RNP complexes that re required to initiate translation. Particle formation is initiated when the coat protein (CP) binds with RNA and is stabilised by a set of proteins at the 5'end of the virion.

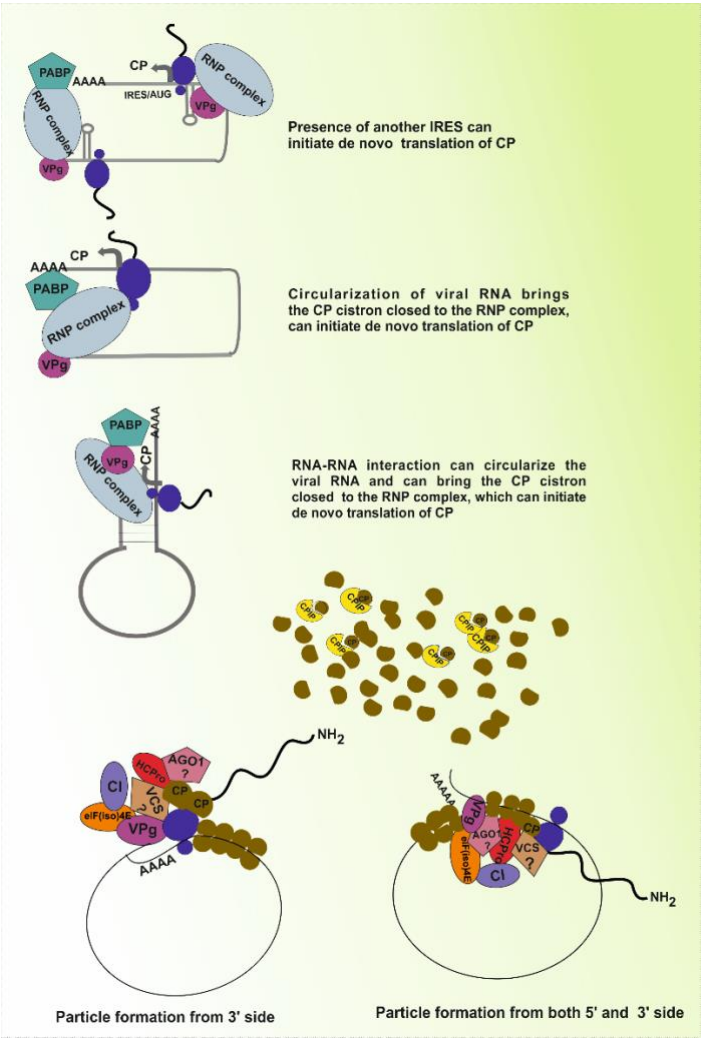


Figure 7: Hypothetical models showing potential mechanisms for coat protein (CP) translation and particle formation. In addition to the translation of CP as part of the polyprotein, three hypothetical alternative models are proposed for CP translation. The first model shows the binding of the translational initiation complex (the RNP complex) to a new internal ribosomal entry site (IRES), which initiates CP translation. The second and third models show that the CP cistron can come closer to the translational initiation complex through PABP-RNP interaction or through RNA-RNA interaction, respectively. Particle formation is initiated from the 3' side of the genome by the binding of the translating *cis* CP with the *trans*-CP. The 5' protein complex is required to stabilise the CP-CP interaction in association with RNA. CP can polymerise over the RNA molecules, either from 3'side to the 5' side or from both directions.

5. CONCLUSIONS AND FUTURE PROSPECTIVE

In most cultivated plants, the VPg–eIF4E/(iso)4E interaction is a fundamental interaction that is required for the successful infection of potyviruses. The mechanism reveals a complex evolutionary strategy, adopted by both the host and the virus, resulting in the interaction being highly variable among host and virus species. Therefore, the specific mechanisms involved for each virus species must be analysed in detail. However, understanding these mechanisms on the molecular level, including the effects of the loss of interaction in any host–virus combination can reveal the various infection strategies employed by potyviruses. In this study, the interaction between the *N. benthamiana* eIF(iso)4E and the PVA VPg was disrupted. This interaction has been proposed to be essential for the assembly of vRNA into PGs, which acts as a safeguard, protecting vRNA from host silencing. This route allows for vRNAs to attach to polysomes and enables protein synthesis. PGs are initiated by HCPro and contain many host and viral proteins. In the presence of the VPg^{mut}, a large number of PG-like foci develop, but these do not contain vRNA, and the mechanisms that result in the development of empty PG-like structures remain unclear. The molecular dissection of such foci will indicate whether additional proteins are involved in the loading of PVA RNA into PGs. These results demonstrated that PVA^{VPgmut} failed to produce particles unless the mutated RNA reverted to the wild-type sequence. Particle formation may require the formation of a complex associated with VPg–eIF(iso)4E. Comparisons of the 5' ribonucleoprotein complex of PVA^{VPgmut} with that of PVA^{WT} RNA may be useful for understanding the components required for particle formation.

The PVA CP, which is expressed from the 3'-most cistron of the PVA genome, accumulates at a different ratio than the other proteins expressed by the PVA genome. VPg has been proposed as the primary component driving enhanced CP expression, which is necessary to fulfil the requirements of adequate particle formation. However, no mechanism has been identified that is capable of quantitatively representing this differential translation from the 5' and 3' sides of the genome. The increased CP accumulation has been proposed to be associated with the inhibition of CP degradation or enhanced CP production. A ribo-seq analysis, examining vRNA at different time points, ranging from the early to late infection stages or in the presence and absence of VPg, would be useful for understanding the mechanisms that regulate this increased CP accumulation in the future. The third study showed that CP requires stabilisation by HCPro to

create a functional virion. Gallo et al. (2018) revealed that replication is associated with virion formation. The replication-deficient virus $PVA^{\Delta GDD}$, which is able to undergo assembly in PGs and undergoes translation, fails to produce particles. The $PVA^{\Delta GDD}$ virus lacks VPg at its 5' end, featuring a cap instead. Therefore, particle formation may require a core complex, consisting of both host and viral proteins, including VPg, eIF(iso)4E, HCPro, AGO1, and VCS, which may allow CP to bind to vRNA for the initiation of particle formation or can provide stability to the particles. However, this hypothesis requires further investigation.

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